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PREFACE

The writing of a preface to one of the *Annual Reviews* provides a welcome opportunity to bring to the attention of our colleagues certain matters specifically relevant to the volume in question and others that are of a more general character.

There is nothing peculiar to this volume that need be written within the preface, unless it be the thanks that we would express to those who have collaborated in the authorship of its 23 chapters. It has been our privilege year by year to convey to our authors the gratitude which we and many others feel for the time and labor extended in preparing the several reviews. While the writing of reviews of one sort or another is becoming a literary and intellectual experience which falls to the lot of almost every scientist sooner or later there is nothing in the accumulated experience that makes the task easier. In these *Reviews* especially it is admittedly arduous. A strenuous effort is made to maintain them at a critical level, a goal that can only be achieved within a reasonable compass by an increasingly rigorous discrimination in the selection of papers to be reviewed. As we mentioned last year this phase of the problem of reviewing is becoming more and more difficult because of the rapid increase in the number of original papers. For better or for worse it is leading to the issuance of more and more publications of the informative abstract and review type.

Years ago, with the introduction of the *Annual Review of Physiology*, we found ourselves convinced that various chapters hitherto appearing in the *Annual Review of Biochemistry* might best be transferred to the new *Review*. The policy by which we were guided consisted in general in the transfer of those subjects that were exclusively devoted to animal biochemistry: "Liver and Bile"; "The Metabolism of Brain and Nerve"; a portion of "Muscle"; "Developmental Physiology or Chemical Embryology," etc. Now, with the problem again before us of utilizing as effectively as possible six dollars' worth of space in the *Annual Review of Biochemistry* we find a possible solution in transferring to the new *Annual Review of Plant Physiology* that portion of the subject matter which is peculiar to plants and soil. We do so with the full realization that biochemistry concerns itself with all living things. The creation of a new *Review* for plants may seem only to contribute to the further division of the sciences into more narrowly restricted fields and to give to biochemistry, as found within the pages of the *Review*, a too narrow definition. It is, however, our hope and confident expectation that all investigations of a fundamental character, whether the subject of research be a plant, an

animal, or a microorganism, will continue to find their place in the *Annual Review of Biochemistry*. Those phases of metabolism that are common to living things generally, researches on enzymes, on biological oxidations, on the descriptive chemistry of the compounds found in living things—these and similar subjects of fundamental import constitute the core of biochemistry. Doubtless many papers of major interest will receive consideration in the two *Reviews*. Within limits, such duplication is desirable and will not be discouraged.

From time to time within the past few years another matter has come to the attention of the Committee and may well deserve the advice of the readers of these *Reviews*: the problem of literature citations. We have for years consistently recommended and employed the system of collecting bibliographical references at the end of each chapter in the order in which they are cited in the text. An alphabetical listing has seldom been followed. The references in the text have frequently been restricted to numbers only, occasionally extended, by wish of the author, to the inclusion of names as well. The method used has economized on space and has greatly facilitated the otherwise tedious problem of preparing the author index for the volume as a whole. The author index, in turn, has served the valuable function of permitting the reader with ease to locate the cited papers of any of the authors. Perhaps too naively, it seems to us that alphabetical listings of literature citations have ceased to be of much value in locating papers. In these days of multiple authorship there is no assurance of finding a paper by Dokes under "D"; it may as likely as not be published with any one of his seven collaborators listed as the first author. The problem, however, appeals to some as of considerable consequence and quite possibly, indeed, there are certain advantages in the alphabetical system of which we are entirely unaware. Since it is our desire to improve steadily the quality and usefulness of the *Reviews* we would welcome the advice of our readers on these and other matters.

To our editorial assistants and other members of the office staff we extend our thanks for their continued interest and conscientious help. We appreciate also the cordial collaboration of our printers, the George Banta Publishing Company.

H. J. A.	J. M. L.
H. J. D.	G. M.
D. R. H.	H. A. S.
H. S. L.	

ERRATA

Volume XVI

page 652, references 28, 30, 31, 32: for *Physiol. Revs.* read
Phys. Rev.

page 654, reference 87: for *Physiol. Revs.* read *Phys. Rev.*

Volume XVII

page 63, reference 113: for *J. Biol. Chem.* 110, 653 (1945) read
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page 413, line 8: *delete* 100 *read* 3 $\mu\text{g.}$ per gm.

page 413, line 9: *delete* 100 *read* 3.6 and 5.1 $\mu\text{g.}$ per gm.

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BIOLOGICAL OXIDATIONS¹

BY PAUL W. PREISLER AND F. EDMUND HUNTER, JR.

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GLYCOLYSIS

Significant contributions concerning the enzyme which catalyzes the one oxidative reaction of glycolysis, the oxidation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid by diphosphopyridine nucleotide (DPN), have appeared. The detailed procedure for preparing crystalline enzyme from rabbit muscle has been published by Cori, Slein & Cori (1). For optimal activity the enzyme must be incubated with cysteine to be converted to the active or "reduced" form. This form is irreversibly inactivated by iodoacetate, while the inactive or "oxidized" form is not inactivated. The turnover number for 100,000 gm. of protein corresponds to a reduction of 6,700 moles of DPN per min. at pH 8.6 and 27°C. Since the turnover number would be considerably lower at the pH of muscle, the high concentration of this enzyme in muscle may be related to its relatively low catalytic activity. Taylor *et al.* (2) have demonstrated that one equivalent of DPN per 50,000 gm. of protein remains rather firmly bound to the rabbit muscle enzyme on repeated recrystallizations. Prolonged dialysis will not remove this DPN, but intestinal phosphatase, or a charcoal such as norit, will remove it without denaturing the protein. The protein moiety has not been altered as indicated by its enzymatic activity when DPN is added. If the coenzyme is removed from several times recrystallized enzyme, it will not recrystallize until DPN is added. The best evidence that the bound DPN may actually function as a prosthetic group is that it is reduced when the enzyme is exposed to substrate. If this tightly bound coenzyme functions as the only prosthetic group, once it is reduced it must be displaced fairly readily by oxidized DPN from the medium, for continuous reduction of DPN added to the medium occurs. The bound DPN, after reduction, will react with pyruvic acid in the presence of lactic dehydrogenase. This observation suggests that the reduced DPN may shift from one enzyme to the other without actually being dis-

¹ This review covers the period from October, 1947 to November, 1948.

placed by the oxidized form. All of the nitrogen in crystalline 3-phosphoglyceraldehyde dehydrogenase can be accounted for by 18 known amino acids and DPN according to Velick & Ronzoni (3).

While there have been reports (4) that glycolysis in the chick embryo was a nonphosphorylating glycolysis, many workers have questioned the concept of a fundamentally different pathway in the embryo as compared with the adult. Novikoff, Potter & Le-Page (5) found that hexosediphosphate was converted to lactic acid. It might have been dephosphorylated before glycolysis, but these workers were able to demonstrate in embryonic tissue all of the known intermediates and coenzymes of the phosphorylating glycolytic scheme. Levy & Young (6) have reported considerably higher DPN levels than Novikoff *et al.*

Lactic acid dehydrogenase from *Mycobacterium phlei* has been reported by Edson (7) to be different from that in animal tissues. Either no pyridine coenzyme is required or it is present in the enzyme in a nondissociable form. A flavin prosthetic group has been suggested. Whether the same enzyme catalyzes aerobically the oxidation of lactate to acetate without the intervention of pyruvate must remain undecided until greater purification is attained. Such an oxidation would be somewhat analogous to that discovered by Ochoa's group, in which malate + triphosphopyridine nucleotide (TPN) \rightarrow pyruvate + CO₂ + TPN red, apparently without the intermediate formation of oxaloacetate.

Racker & Krimsky (8) have found that the inhibition of glycolysis in brain homogenates by ferrous sulfate is apparently due to activation of a factor in the tissue, and that this factor produces its effect by partially inactivating 3-phosphoglyceraldehyde dehydrogenase. Meyerhof (9) has summarized the major contributions in the study of glycolysis from 1940 to 1948.

TRICARBOXYLIC ACID CYCLE

An extensive study on reactions of the tricarboxylic acid cycle by Green, Loomis & Auerbach (10) has demonstrated that all of the essential reactions of the cycle can be shown in a single tissue preparation. Because of improved methods and some new techniques of isolating individual reactions, these workers were in general able to make a more clear-cut demonstration of the individual reactions than Krebs was able to do in his original work on which the cycle is based. All of the enzymes necessary to carry out the

complete cycle are present in the insoluble particles from tissue homogenates. There is reason to believe that this group of enzymes exists and functions as a particulate complex in the cytoplasm of cells. Kennedy & Lehninger (11) state that intact mitochondria catalyze the reactions of the tricarboxylic acid cycle, a fact also discussed by Schneider (12). The oxidation of carbohydrate in plants involves the tricarboxylic acid cycle according to Bonner (13).

PYRUVIC ACID OXIDATION

Attempts to demonstrate the exact intermediates between pyruvic acid and isocitric acid have continued. Gurin, Delluva & Wilson (14) report that radioactive lactate, given to phlorhizinized animals, is converted in considerable amounts to acetoacetate. This work confirms earlier experiments, isotopic and nonisotopic, which indicate that pyruvate is converted to a two-carbon intermediate before it enters into metabolic reactions, but the identity of the intermediate remains to be established.

Work with fluoroacetate as an inhibitor also has given support to the belief that pyruvate is converted to a two-carbon intermediate. Bartlett & Barron (15) feel that the effect of fluoroacetate is due to blocking of oxidation of acetate formed during pyruvate oxidation. One might also take the view that the inhibitor blocks further reaction of a two-carbon intermediate which breaks down to free acetic acid and accumulates as such. Whichever way this question is finally decided, the observation that fluoroacetate decreased acetoacetate formation from fatty acids (presumably through a two-carbon intermediate) but considerably increased acetoacetate formation from acetate must be explained, for these results and the observation that fluoroacetate does not inhibit acetylations suggest that it does not block all reactions into which acetate or a two-carbon intermediate enters. While Kalnitsky & Barron (16) believe that the fluoroacetate inhibition is due to direct competition with acetate, Black & Hutchens (17) suggest that secondary effects must also be considered. Webb & Elliott (18) report that very little acetate accumulates during pyruvate oxidation in brain suspensions, even when fluoroacetate is added.

Kalnitsky & Barron (19, 20) report that fluoroacetate markedly increases the amount of citrate formed from oxaloacetate by kidney homogenates, and that a significant part of the effect must be

due to increased formation, not decreased removal. It seems paradoxical that fluoroacetate should increase citrate formation when one of its effects seems to be blocking entrance of acetate into the tricarboxylic acid cycle. Whether these data mean that oxaloacetate can form citrate without intermediate formation of pyruvate and a two-carbon intermediate remains to be determined. Kalnitsky (19) states that the only effect of barium ion in increasing citrate accumulation is to decrease citrate removal, but Krebs & Eggleston (21) do not entirely agree.

Baer (22) reports on some properties of the cell-free pyruvic oxidase preparation from *Proteus vulgaris* (pyruvate→acetate). The protein part of the enzyme is inactivated by several reagents which react with sulfhydryl groups, and this inhibition can be reversed by British Anti-Lewisite (BAL) in most cases. However, if the essential and firmly bound prosthetic group, thiamine pyrophosphate, has not been removed by acid treatment, the sulfhydryl reagents are without effect, suggesting that the diphosphothiamine may be linked to the protein through essential sulfhydryl groups.

The protein part of the enzyme responsible for the first step in pyruvic acid oxidation is formed when *Streptococcus faecalis* organisms are grown on a synthetic medium, but an essential accessory factor must be supplied by yeast extract before the system is active, according to O'Kane & Gunsalus (23). This factor seems to be different from known co-factors, including coenzyme A.

Although acetaldehyde and acetate may not actually be intermediate in the oxidation of pyruvate in many cases, they probably enter the metabolic cycle at the same point, so it is worthy of note that Stadtman & Barker (24) find that aerobically acetaldehyde is oxidized to acetyl phosphate by an enzyme preparation from *Clostridium kluyveri*, while anaerobically it undergoes dismutation with the formation of acetyl phosphate and alcohol. Acetate metabolism has been reviewed by Bloch (25). Nord & Vitucci (26) have studied the unusual metabolic pathway in molds, whereby acetate is converted to oxalic acid. Lynen (27) has used deuterium-labelled acetate to follow the metabolism in yeast, concluding that no symmetrical tricarboxylic acid participates. However, Ogston (28) has recently pointed out that such a conclusion may not be justified. Weinhouse & Millington (29) have followed acetate metabolism in yeast with C^{13} . Their data confirm present day concepts about the tricarboxylic acid cycle, and in addition the iso-

topic distribution indicates that not all four carbon dicarboxylic acids are derived from citrate or isocitrate. Some result from other pathways of acetate metabolism in yeast, possibly direct condensation of two molecules. Karlsson & Barker (30) find that adaptive enzymes for the oxidation of intermediates of the tricarboxylic acid cycle are readily formed in *Azobacter agilis*, yet acetate is readily metabolized without the appearance or detectable presence of these enzymes, a fact which indicates that acetate probably is not oxidized through the cycle in this organism.

EFFECT OF VITAMIN DEFICIENCIES ON PYRUVIC ACID OXIDATION

Thiamine.—The removal of pyruvate in heart muscle slices by reduction to lactate and by oxidation has been studied by Olson *et al.* (31). Deficiency in thiamine decreases removal of pyruvate over oxidative pathways and probably does not alter reduction to lactate. The rate of pyruvate oxidation by deficient tissues is closely correlated with thiamine content. Addition of thiamine *in vitro* to deficient duck heart slices stimulates oxidation of pyruvate. These authors found marked differences in the relationships between oxygen consumption, pyruvate reduction, and pyruvate oxidation depending on species, fasting, pair feeding, and *ad libitum* feeding.

Biotin.—The same group, Olson *et al.* (32), has also studied pyruvate metabolism in biotin deficient ducks. In heart slices the oxidative removal of pyruvate was 48 per cent below normal, and the oxygen consumption was correspondingly low. Biotin added to tissue *in vitro* had no effect, but if given to the living animal it brought pyruvate metabolism to normal in 24 hr. Since the formation of carbon dioxide from succinate was depressed as much as or more than pyruvate oxidation, the authors consider that the defect in pyruvate metabolism in biotin deficiency may be at the oxaloacetic acid carboxylase step. That there may be more than one metabolic alteration is suggested by the observation that reduction of pyruvate to lactate is markedly decreased by biotin deficiency in contrast to no change in thiamine deficiency.

Pantothenic acid.—Since Novelli & Lipmann (33) reported increased pyruvate oxidation when pantothenic acid was supplied to bacteria deficient in the vitamin, several groups of workers have been investigating the exact role which pantothenic acid may play

in pyruvate metabolism. Essentially all the pantothenic acid in tissues seems to be present in a form that behaves like coenzyme A (34). Hegsted & Lipmann (35) have presented some figures on the amount of pantothenic acid in partially purified coenzyme A. The parallelism between pantothenic acid content and coenzyme A activity leaves little doubt that pantothenic acid is part of the coenzyme. Olson & Kaplan (36) found that the coenzyme A content of liver tissue of ducks fell fairly rapidly to about 40 per cent of normal when the animals were placed on a diet deficient in pantothenic acid. *In vitro* addition of the vitamin resulted in a small increase of coenzyme A, while *in vivo* administration resulted in marked increases in coenzyme A within two to four hours. Ability to oxidize pyruvate was closely correlated with the coenzyme A content of the tissue.

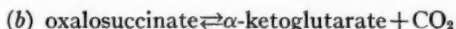
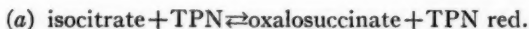
Although there have been indications from the earliest work with coenzyme A that it was involved in the metabolism of acetate or the two-carbon intermediate formed from pyruvate, the question as to whether it was also involved in the conversion of pyruvate to two-carbon compounds remained open. Riggs & Hegsted (37) have observed that administration of acetate does not increase acetylation of *p*-aminobenzoic acid in normal rats, but does cause considerable increase in pantothenic acid deficient animals. This suggests that the supply of metabolic acetate is low in pantothenic deficient animals and that coenzyme A may function in formation of acetate from pyruvate. Somewhat more specific and conclusive evidence on this point is available from the preliminary data of McElroy & Dorfman (38) on bacteria, which suggest that pantothenic deficient organisms decarboxylate pyruvate, but do not oxidize it further like normal organisms do. Since acetylmethylcarbinol accumulates, presumably the two-carbon intermediate, perhaps acetaldehyde, condenses to form this four-carbon molecule. These workers conclude that coenzyme A is involved in the oxidation of acetylmethylcarbinol or some closely related substance. It would seem possible that the coenzyme is involved in the oxidation of the acetaldehyde to acetate, and that acetylmethylcarbinol may be formed only when oxidation of the intermediate does not occur.

Some major contributions of the year have been the studies on the role played by coenzyme A in acetate oxidation. Novelli & Lipmann (39) found that yeast which was deficient in pantothenic

acid and coenzyme A oxidized acetate only half as rapidly as normal yeast. The authors favor the view that the principal role of coenzyme A is in the primary attack on acetate (and the two carbon intermediate from pyruvate), enabling it to undergo condensation with oxaloacetic acid to enter the tricarboxylic acid cycle. Kaplan & Lipmann (34) have demonstrated the widespread distribution of coenzyme A. Tissues and microorganisms which are generally known to use acetate groups in the synthesis of numerous compounds, such as fatty acids, acetoacetate, and cholesterol, contain a much higher coenzyme content than tissues such as muscle, where the major pathway for acetate may be oxidation by way of the tricarboxylic acid cycle. Since most of the work on the effect of coenzyme A on pyruvate and acetate oxidation has been done on microorganisms and yeast, where alternative pathways may exist, it may take a direct study of the conversion of acetate and pyruvate to citrate to demonstrate that coenzyme A is important for the condensation of acetate and oxaloacetate.

ISOCITRIC ACID OXIDATION

The oxidation of isocitric acid to α -ketoglutaric acid involves oxalosuccinic acid as an intermediate. Ochoa (40) has demonstrated that the two steps, (a) and (b), are both enzymatic and both reversible.



He achieved a partial purification of isocitric dehydrogenase and confirmed earlier reports of others that this enzyme is strictly specific for TPN (triphosphopyridine nucleotide). The enzyme catalyzing the second reaction, decarboxylation of oxalosuccinic acid, was studied by Ochoa & Weisz-Tabori (41). Manganese ion, but not magnesium ion, serves as a co-factor. Oxalosuccinic decarboxylase and oxaloacetic decarboxylase apparently are separate enzymes, each specific for its own substrate. However, the question as to whether isocitric dehydrogenase and oxalosuccinic decarboxylase are separate enzymes or two activities of a single enzyme remains open, for no separation of the two has been achieved, and isocitric acid is strongly inhibitory to the decarboxylation of oxalosuccinic acid. Ochoa points out that the situation may be analo-

gous to the malic acid oxidizing enzyme of pigeon liver reported earlier (42). In addition Ochoa (40) demonstrated how these reactions could play an important role in carbon dioxide fixation. Although the equilibrium of reactions $a+b$ is far to the right, if the oxidized TPN is continuously reduced by some other enzymatic system the reactions readily proceed to the left.

The conclusions of Ochoa concerning the reversibility of the oxidation of isocitric acid to α -ketoglutaric acid have been confirmed by Grisolia & Vennesland (43), using radioactive carbon. The same group (44) has reported the presence of this enzyme system in plants and observed a stimulating effect of adenosinetriphosphate (ATP) on carbon dioxide fixation.

α -KETOGLUTARIC ACID OXIDATION

Ajl & Werkman (45) report that the oxidation of α -ketoglutarate to succinate and carbon dioxide is reversible, as indicated by isotope experiments with an enzyme preparation from *E. coli*.

SUCCINIC ACID OXIDATION

Incubation of kidney tissue particles at 38°C. for 15 min. before adding substrate destroyed other enzymes so that the step succinate \rightarrow fumarate + malate could be isolated quantitatively in the work of Green, Loomis & Auerbach (10). Krebs earlier had used arsenite for this purpose.

Studies on the location of the succinoxidase system within the cell have been continued by Hogeboom and co-workers. Succinoxidase was found (46) almost exclusively in the "large granule" fraction from liver homogenates. Since these granules were the size of mitochondria, yet did not behave histologically like intracellular mitochondria, this group of workers (47) extended their work by devising a method for isolating morphologically intact mitochondria. Using this preparation they were able to conclude with greater certainty that the mitochondria contained 65 to 82 per cent of the succinoxidase in the cells, probably all of the cytoplasmic succinoxidase.

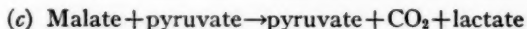
Dietary factors may influence the succinic dehydrogenase activity of liver according to Byerrum, Erway & DuBois (48). Novikoff & Potter (49) report that formation of succinoxidase, cytochrome reductase, malic dehydrogenase, and oxaloacetic oxidase lag behind regeneration of total mass of liver tissue after partial

hepatectomy. Normal values are reached within three or four days. Tipton & Nixon (50) have shown that the depressing effect of thiouracil feeding on liver succinoxidase and cytochrome oxidase is entirely due to effects on the thyroid gland and is not a direct action of the drug. The succinoxidase system from several tissues is inhibited *in vitro* by estrone and by compounds related to diethylstilbestrol in low, but perhaps not physiological, concentrations according to McShan, Meyer & Erway (51). These workers and Case & Dickens (52) agree that the inhibitory effect is not correlated with estrogenic activity, but rather is associated with the presence of phenolic hydroxyl groups. Thyroxine (51), although it increases activity *in vivo*, causes inhibition when added to the isolated system in a 10^{-4} M concentration. McShan's group has presented fairly convincing evidence that the effect of synthetic estrogens is entirely on the cytochrome oxidase part of the system. However, Case & Dickens report that *p-p'*-dihydroxystilbene in low concentrations totally inhibits the oxidation of succinate, but is without effect upon either cytochrome oxidase or succinic dehydrogenase. They postulate an effect on the intermediate link between the two enzymes. The succinic dehydrogenase part of the system is markedly inhibited *in vivo* by several bacterial endotoxins according to Kun & Miller (53).

MALIC ACID OXIDATION

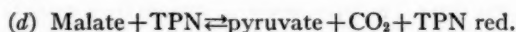
When malate oxidation by washed kidney particles is carried out at pH 9.0 instead of pH 7.5, the oxidation proceeds only one step, as discovered by Green and co-workers (10). These authors report that cyanide traps the oxaloacetate before decomposition, so that oxygen consumption occurs, but no carbon dioxide is evolved. Methylene blue must be added as an electron carrier when cyanide is present.

While in the above instance the oxidation of malate is presumably carried out by the malic dehydrogenase which has been recognized for many years, Mehler *et al.* (54) have found a different malic acid oxidizing enzyme in pigeon liver. This enzyme was discovered in a study of the dismutation represented by reaction (c).



Unable to reconstruct a system which would catalyze reaction (c) from purified malic dehydrogenase; lactic dehydrogenase, a par-

tially purified oxaloacetic carboxylase, and coenzymes, Ochoa's group concluded that they were dealing with a new enzyme which reversibly catalyzes the oxidative decarboxylation of malic acid, reaction (d).



Combined with lactic dehydrogenase the preparation catalyzes reaction (c) in both directions. This enzyme requires TPN as its coenzyme, a property which distinguishes it from the malic dehydrogenase studied so far in most tissues. By combining the system with glucose-6-phosphate dehydrogenase to convert oxidized TPN back to the reduced form, it was clearly demonstrated how this enzyme could function in carbon dioxide fixation. All of the evidence so far indicates that one single enzyme or inseparable enzyme complex catalyzes the reaction, without oxaloacetic acid as an intermediate. Oxaloacetate is not reduced to malate; neither is it possible to detect it when malate is oxidized. The decarboxylation of oxaloacetate is catalyzed by the enzyme, and since it has not been possible to separate this activity by purification (55), it is concluded that it may well be due to the same enzyme. TPN, essential for malic acid oxidation, is stimulatory for decarboxylation. Biotin may bear some relation to the activity of this enzyme (56). Lwoff & Ionesco (57) report that potassium ions are necessary for the direct conversion of malate to pyruvate by *Moraxella lwoffii*. Korkes & Ochoa (58) find that *Lactobacillus arabinosus* adaptively forms a malic acid oxidizing enzyme similar to that of pigeon liver except that it seems to be DPN instead of TPN specific.

COENZYMES

The coenzyme specificity of several enzymes has been checked by Mehler and co-workers (54), who demonstrated or confirmed that isocitric dehydrogenase is TPN specific, 3-phosphoglyceraldehyde dehydrogenase is DPN specific, while glutamic dehydrogenase reacts equally well with either coenzyme. Malic dehydrogenase reacts more rapidly with DPN and lactic dehydrogenase reacts very much more rapidly with DPN, though both of these enzymes can react with TPN.

Because of the widespread use of spectrophotometric methods in following oxidation-reduction reactions involving DPN and TPN, the exact value for the extinction coefficient at 340 $m\mu$ for

the reduced form is of considerable importance. Horecker & Kornberg (59) have materially helped to clear up this question by permitting known amounts of substrates to react with the pyridine nucleotides in enzymatic reactions which, for practical purposes, may be considered as going to stoichiometric completion. The calculated extinction coefficients, 6.22×10^4 cm²/mole for reduced DPN and TPN, are very close to that reported many years ago by Ohlmeyer.

A new reducing agent, sodium borohydride (NaBH₄), has been proposed by Mathews (60) for chemically reducing DPN and TPN in determinations based on absorption of the reduced form at 340 mμ. It has the advantage over sodium hydrosulfite that it does not absorb at 340 mμ itself. However, the new reagent cannot be used to prepare reduced coenzymes for enzymatic experiments, for only about half of the reduced material reacts. The identity of the other reduced product is uncertain. Sumner & Krishnan (61) have published an enzymatic method of determining DPN, using either lactic dehydrogenase or phosphoglyceraldehyde dehydrogenase.

The splitting of DPN which may reduce its concentration to a limiting level in respiration studies *in vitro* is apparently due to two enzymes. One splits off the nicotinamide group and is, therefore, a nucleosidase. The other enzyme, described by Kornberg & Lindberg (62), causes a pyrophosphatase type splitting in the middle of the molecule. Unlike the first enzyme, the latter is not inhibited by nicotinamide.

COUPLING PHOSPHORYLATION WITH OXIDATIONS

A review has been published by Ogston & Smithies (63) in which they critically consider the question of the maximum number of energy rich phosphate bonds which can be created for each atom of oxygen consumed. Unfortunately, their conclusion that phosphorus to oxygen values even as high as 2.0 seem unlikely at an appreciable rate of reaction is based on several assumptions not entirely justified on the basis of our present knowledge. That they may be mistaken in assuming that only the oxidizing potential between cytochrome-*c* and substrate level is tapped for conversion into phosphate bond energy has already been pointed out by Weil-Malherbe (64). While 16,700 calories is close to the value for the phosphate bond energy of phosphocreatine on the basis of informa-

tion available, it should not be assumed that 16,700 calories, or a potential difference of 0.36 volt, is essential for the generation of every phosphate bond. Most high energy phosphate bonds may be created at about the level of those in ATP, perhaps 12,000 to 13,000 calories, and are used directly to phosphorylate glucose and provide energy for metabolic reactions without intervention of phosphocreatine, which seems merely a side reaction in equilibrium with ATP. While Ogston & Smithies have pointed out a number of puzzling things which must be explained by further experimental work, it is difficult to accept their view that Ochoa's directly observed values of 1.6 to 1.8 for phosphorus to oxygen ratios should be accepted without correction for adenosinetriphosphatase activity. Even a modest correction would indicate true ratios of 2.0 or more. [Furchgott & Shorr (65) have reported low values for phosphorus to oxygen ratios with succinate oxidation in cardiac and smooth muscle, but with the type of preparation used many side reactions make quantitative evaluation virtually impossible. Their experiments strikingly illustrate the fact that the yield of phosphate bonds per atom of oxygen is lower with succinate as substrate than with glucose as substrate, a difference indicated by data of earlier workers.] More recently the use of washed tissue particles has reduced the number of side reactions and permitted the direct observation of phosphorus to oxygen ratios of appreciably more than 2.0 in α -ketoglutarate oxidation [Hunter (66), Knox & Green (67)]. The only published value on washed tissue particles, 2.2, is reported by Loomis & Lipmann (68), who used glutamate as substrate. Hunter (69) has found that approximately one molecule of phosphate is esterified per molecule of α -ketoglutarate oxidized when oxaloacetate is the oxidant, with no correction applied for phosphatase activity. This is compatible with a phosphorus to oxygen ratio of at least 2.0.

Friedkin & Lehninger (70) have reported that incorporation of radioactive phosphate into the acid soluble organic ester fraction of phosphate compounds is greatly increased when reduced DPN is added as substrate to washed liver tissue particles, as would be expected if oxidations involving the transport of electrons from the pyridine nucleotides to oxygen were coupled with phosphorylation at one or more steps. Further study of these oxidation steps should demonstrate eventually the true phosphorus to oxygen ratios and the points at which the energy rich phosphate bonds are generated.

The oxidation of β -hydroxybutyrate to acetoacetate also causes phosphorylation. Presumably this is equivalent to oxidation of reduced DPN.

Although it has not been possible to demonstrate any net incorporation of inorganic phosphate into organic form during fatty acid oxidation, Lehninger & Kennedy (71) believe that maintenance of ester phosphate at a constant level and incorporation of radioactive phosphate into the organic ester fraction indicate that at least part of the free energy released during fatty acid oxidation is converted into phosphate bond energy. Data on oxygen consumption and acetoacetate formation are available for only one of these experiments, but they suggest that a little of the material may have been oxidized in the tricarboxylic acid cycle. Thus the coupling of phosphorylation with the oxidation of octanoate to acetoacetate remains to be clearly demonstrated experimentally. Since most of the energy release from fatty acids occurs during oxidation of the two-carbon fragments through the tricarboxylic acid cycle, phosphorylation due to the complete oxidation of fatty acids is to be expected.

Previous *Annual Reviews of Biochemistry* have presented discussions of the factors causing interference with the coupling of phosphorylations with oxidations. Spiegelman *et al.* (72) have published data indicating that azide interferes with the transfer of phosphate from 1,3-diphosphoglyceric acid to the adenylate system rather than the coupling of phosphorylation with the oxidation of triosephosphate.

The effect of dinitrophenol (DNP) on the oxygen consumption of brain slices and homogenates has been studied by Peiss & Field (73), who suggest that this substance removes an inhibiting or regulating factor normally active in intact cells but not functioning in homogenates. More recently Loomis & Lipmann (68) reported that low concentrations of DNP reversibly inhibit coupling of phosphorylation with glutamate oxidation in washed kidney tissue particles. Such a dissociation of phosphorylation from oxidation would explain the known effects of dinitrophenol on respiration and metabolism. The mechanism appears to be through a replacement of phosphate as an obligatory component of the oxidation system, an effect somewhat analogous to that of arsenate with triose phosphate dehydrogenase. Quinacrine (atabrine) seems to have an effect just like that of DNP. Knox, Noyce & Auerbach

(74) refer to the work of Taggart in showing that dinitrophenol and gramicidin cause dissociation of phosphorylation from oxidations of the tricarboxylic acid cycle.

The work of Hummel (75) indicates that oxidation of substrates remains normal in dystrophic muscle from vitamin E deficient guinea pigs, while phosphorylation of creatine is greatly diminished, indicating a dissociation of phosphorylation from oxidation. α -Tocopherol phosphate added *in vitro* has no effect on phosphorylation or oxidation (76). The exact role of the vitamin in muscle metabolism is not known, for different results are observed with hamsters and rabbits.

Shapiro (77) suggests that low concentrations (0.001 *M*) of phlorhizin inhibit the phosphorylation coupled with oxidations in the tricarboxylic acid cycle, but since the oxidation of pyruvate and citrate are inhibited by the same concentration the effect may primarily involve the oxidations, not the coupled phosphorylations. Meyerhof & Wilson (78) suggest a selective inhibitory action of phlorhizin on transfer of phosphate from phosphopyruvate to the adenylate system. However, the concentration required is 10 times as great as that which blocked the tricarboxylic acid cycle in Shapiro's experiments.

Changes which cause dissociation of phosphorylation from oxidation occur during preparation and aging of tissues. Potter, LePage & Klug (79) have found that in isotonic homogenates of tissues the oxidation of oxaloacetate is maintained for some time and phosphorylation occurs, while with water homogenates the initial rate of oxygen uptake is almost equally high but the ATP is rapidly depleted and never rebuilt. Friedkin & Lehninger (70) report that aging tissue preparations two days in the cold does not alter the oxidation of β -hydroxybutyrate, but completely eliminates the phosphorylation which is coupled to this oxidation in fresh preparations.

FATTY ACID OXIDATION

Lipoxidase.—Bergström & Holman (80) have reviewed work on this enzyme up to May 1948. Crystalline enzyme has been prepared from soybeans. Apparently iron or an iron-containing prosthetic group is not a part of the enzyme molecule. Smith & Sumner (81) find that when methyl linoleate and bixin (a carotene derivative) are exposed to lipoxidase, a proportionality exists between the

increase in absorption at $232\text{ m}\mu$ due to methyl linoleate oxidation and the decrease in absorption at 455 and $480\text{ m}\mu$ due to bixin oxidation, suggesting a coupled oxidation reaction. Smith & Sumner (82) find that the reported activation of lipoxidase by a polypeptide from soybeans was not seen under their conditions. Holman (83) suggests that the normal function of the enzyme in germinating soybeans may be to initiate oxidation of linoleic and linolenic acids, which then can proceed automatically.

Fatty acid dehydrogenase.—Burton (84) has studied the fatty acid dehydrogenase which introduces a double bond near the center of the carbon chain of higher fatty acids. He demonstrated that both the enzyme from ox liver described by Annau and that studied in rat liver by Lang & Mayer can be activated either by adenylic acid or hypoxanthine. Since hypoxanthine is a better activator than adenylate, Burton suggests that adenylate may exert its effect through hypoxanthine formed from it. The enzyme seems to require DPN (or some substance in 45 per cent pure DPN), even when the other activators are present. Champougny & Le Breton (85) have also studied this enzyme, continuing from their earlier work in which they concluded that nicotinamide, pyridoxine, and pantothenic acid can all act as activators. That so many diverse substances can serve as activators for one enzyme seems unlikely. Champougny & Le Breton report that partially purified enzyme is inactive even in the presence of activators, but that the addition of purified alkaline phosphatase restores full activity. This puzzling phenomenon must be confirmed and explained by further work.

Fatty acid oxidation by washed tissue particles.—Lehninger & Kennedy (71) have found that liver tissue particles require three substances, cytochrome-*c*, neutral salts or sucrose, and catalytic amounts of malate or oxaloacetate for fatty acid oxidase activity, in addition to the adenine nucleotide and magnesium ion requirements earlier demonstrated. The neutral salts or sucrose seem to be important for maintaining the complex of enzymes in the proper physical state. The oxaloacetate (malate) appears to have two effects: (a) initiation of the oxidation of fatty acid, with the exact mechanism not understood; and (b) condensation with the two carbon fragments formed from the fatty acid so that the tricarboxylic acid cycle goes into operation. Additional experiments have demonstrated that inorganic phosphate is essential. Since a

number of enzymes must be acting, it is not possible to know whether the requirements for phosphate, adenylate, magnesium ion, and cytochrome-*c* are essential for the malate oxidation and activating effect, for the fatty acid oxidizing enzymes, or for both. It is possible that these requirements may apply to both parts of the system. Certainly they are already known to be essential for the tricarboxylic acid cycle to metabolize the two-carbon fragments. The isolation of mitochondria by Kennedy & Lehninger (11) and by Schneider (12) has permitted demonstration that essentially all of the fatty acid oxidase system of the liver cell is in the mitochondria.

Washed kidney tissue particles, supplemented with ATP, magnesium ion, inorganic phosphate, and a small amount of pig heart extract, have been shown by Grafflin & Green (86) to oxidize fatty acids completely to carbon dioxide and water, corresponding in general to the system studied in liver by Lehninger and co-workers. Grafflin & Green tested a considerable range of substrates. α - β Unsaturated, β -hydroxy, β -keto acids, and other substances which might be intermediates in β -oxidation are in general oxidized as well as the parent compound, while substances in which β -oxidation might be expected to be blocked by substitutions are in general not oxidized. Acetoacetate is oxidized by the rabbit kidney enzyme and to some extent also by rabbit liver, while the rat liver enzyme of Lehninger does not oxidize acetoacetate. Other evidence which indicates a difference between the rat and the rabbit liver is that odd-numbered fatty acids do not give rise to acetoacetate in the rabbit liver system. It has been suggested that acetoacetate might come only from butyrate in rabbit liver. This would mean that two-carbon fragments formed from fatty acids can condense to acetoacetate in rat liver but not in rabbit liver. Atchley (87), using the counter current distribution technique, has been able to identify the major products of valeric and isocaproic oxidation as propionic and isobutyric acids, thus confirming β -oxidation and the splitting off of a two-carbon fragment. The mechanism whereby part of the isobutyric acid formed is further oxidized to propionic acid is discussed.

Knox, Noyce & Auerbach (74) have published a detailed study on the stimulating effect of pig heart extract. They find that oxidation of small amounts of any one of several substrates in the tricarboxylic acid cycle will initiate the oxidation of the fatty acid,

which otherwise will not proceed at all. Although oxidation of these "sparking" agents is known to produce phosphorylation, and the "sparking" effect is eliminated when dinitrophenol or gramicidin is used to dissociate phosphorylation from oxidation, their effects cannot be duplicated with ATP or acetyl phosphate.

Acetoacetate metabolism.—Floyd, Medes & Weinhouse (88) have used acetoacetate containing heavy carbon to demonstrate that considerable amounts of acetoacetate can be converted to citrate anaerobically by kidney, thus reaching the same conclusion drawn earlier by Hunter & Leloir (89), who also suggested that the conversion did not occur anaerobically except when linked with a dismutative oxidation of α -ketoglutarate. In both of these studies excess oxaloacetate was used. The use of smaller amounts of oxaloacetate in the experiments of Krebs & Eggleston (90) might explain why the anaerobic conversion did not occur in sheep heart. Comprehensive balance experiments of the aerobic metabolism of acetoacetate in sheep heart and kidney (21) completely confirm the fact that acetoacetate is metabolized through the tricarboxylic acid cycle.

Omega oxidation of fatty acids.—Verkade, Van der Lee & Elzas (91) find that feeding the triglyceride, triundecylin, results in excretion in the urine of long chain dicarboxylic acids.

Reviews.—Breusch (92) and Leloir (93) have each reviewed work on fatty acid oxidation. Gurin (94) has reviewed the work with isotopic tracers, including carbohydrate metabolism as well as fat metabolism.

MISCELLANEOUS ENZYMES

Glyoxalase.—While carrying out a partial purification of this enzyme, Hopkins & Morgan (95) discovered that they could separate a protein-like material which had no glyoxalase activity itself, yet, when added back to the purified glyoxalase, doubled its activity. This substance does not replace glutathione, already recognized as an activator. In fact the new co-factor depends on the simultaneous presence of glutathione for its activating effect.

Alcohol dehydrogenase.—The crystallization of this enzyme from horse liver has been reported by Bonnichsen & Wassen (96). The turnover number reported, 220, seems very low.

Ascorbic acid oxidase.—Within the scope of the series of substrates tested, Dodds (97) finds that this enzyme in cucumbers is

specific for substrates having the dienol group adjacent to a carbonyl group and in a ring structure, but a methylene group can be substituted for oxygen in the ring.

Sugar oxidases.—Detailed information on the physical properties of the enzyme and some of the kinetics of the reaction for glucose oxidase (notatin) of *Penicillium notatum* have been published by Keilin & Hartree (98). They confirm that alloxazine adenine dinucleotide is the prosthetic group, that hydrogen peroxide is formed, and suggest a molecular weight of 150,000. The same authors (99) describe the use of the purified enzyme for a specific manometric determination of glucose.

The glucose oxidase system of liver has been studied by Eichel & Wainio (100), who find that methylene blue, Straub's flavoprotein, or cytochrome-*c* plus cytochrome oxidase will serve as links to oxygen. No diaphorase type of flavoprotein need be added, but the preparation used probably contains some flavoprotein. Whether further oxidation of gluconate goes eventually through the pyruvate state has been questioned (101) on the basis that in thiamine deficient livers pyruvate accumulates during glucose oxidation but not during gluconate oxidation.

The direct oxidation of lactose and maltose to lactobionic acid and maltobionic acid by *Pseudomonas graveolens* has been rather clearly demonstrated by Stodola & Lockwood (102). Reiner & Spiegelman (103) report the oxidation of galactose by yeast which cannot ferment this sugar.

Xanthine oxidase and xanthopterin oxidase.—Kalckar & Klenow (104) report a method for partial purification of xanthopterin oxidase from milk. At first these workers reported that pteroylglutamic acid inhibited xanthopterin oxidase of milk and liver and xanthine oxidase (source not specified), but further work and communication with Lowry & Bessey, who have been studying some of the same reactions, indicated that the inhibition was due to the presence of small amounts of 2-amino-4-hydroxy-6-pteridyl aldehyde as an impurity. This aldehyde has been shown by Kalckar's group (105) and by Lowry (106) to be a powerful inhibitor of the enzyme. Both groups (104, 106) have found that the inhibitor is gradually converted to a noninhibitory substance by the enzyme. The answer to the question whether one enzyme or several different enzymes are responsible for the activity on xanthine, xanthop-

terin, 2-amino-4-hydroxy-pteridine, and the inhibitory aldehyde must come from further work.

Amino acid and amine oxidases.—Krebs (107) has summarized the data on D- and L-amino acid oxidases. The effects of several inhibitors on these enzymes have been studied by Bartlett (108), Klein & Olsen (109), and Symul (110). Activation of D-amino acid oxidase by blood serum has been reported by Wiss & Klingler (111). The activity of amine and amino acid oxidases in different tissues has been investigated by Steensholt (112), Lushinsky & Singher (113), Featherstone & Berg (114), Knight (115), and Cedrangolo (116). Ling & Tung (117) report that the enzyme which catalyzes oxidative demethylation of N-methyl amino acids with the formation of formaldehyde is a flavoprotein of low substrate specificity. Walser (118) states that the best histaminase preparations are impure, do not attack the imidazole ring, but do act on several diamines.

Respiration of different tissues.—A group working with Barron (119, 120) has published general studies on the respiration and metabolic reactions of bone marrow and of lung, two tissues which have not been widely studied.

OXIDATION OF STEROID HORMONES

Estrogen inactivation by liver *in vitro* requires aerobic conditions or a suitable electron acceptor, as indicated by the work of DeMeio *et al.* (121), who also noted a stimulatory effect of DPN in homogenates. Coppedge *et al.* (122) report that the addition of DPN to liver mince enhances or restores its ability to inactivate α -estradiol, suggesting that the electrons removed from the substrate may pass through the pyridine nucleotide system. That cytochrome-*c* is involved in the electron transport from estradiol as substrate was earlier shown by Levy (123). Sweat & Samuels (124, 125) find that DPN is important for inactivation of testosterone by liver minces. The reaction stimulated by DPN results in oxidation of testosterone to 17-ketosteroids, a 75 per cent recovery being possible. When citrate is added instead of DPN, the removal of the hormone is greatly stimulated, but no 17-ketosteroid accumulates. The citrate effect appears not to be due to removal of calcium ion or a general increase in oxygen consumption. Whether DPN and citrate are involved in consecutive reactions or in two separate

pathways is yet to be determined. Clark, Kochakian & Labotsky (126) find that although Δ^4 -androstenedione-3,17 is the chief substance formed from testosterone by liver slices, when this 17-ketosteroid itself is added, reduction back to testosterone seems to be the principal reaction taking place. This suggests that it might not be an intermediate in the conversion of testosterone to androsterone. Schneider & Mason (127) found that dehydroisoandrosterone added to rabbit liver slices is about half reduced at the 17-position to yield a hydroxyl group in place of the ketone group. Much smaller yields of a steroid with hydroxyl groups in both the 16- and 17-positions were found. With androsterone or with etiocholan-3-(α)-ol-17-one as substrate, the same workers (128) found that much more of the compound was reduced at the 17-position, yielding a 3,17-diol, than was oxidized at the 3-position to yield a 3,17-dione. In the case of androsterone some of this dione was reduced to isoandrosterone.

METALLOPROTEIN ENZYMES AND CARRIERS

Cytochrome oxidase.—A soluble and active cytochrome oxidase has been prepared by Wainio *et al.* (129) by adding 3 to 4 per cent sodium desoxycholate to a Keilin & Hartree oxidase preparation from lamb heart and centrifugating at 20,000 \times g for two hours. The enzyme was found in the supernatant liquid, with activity about 2.5 times that of the original K and H material. Partial purification, resulting in a five times more active solution, was accomplished by first treating the K and H oxidase with 2 per cent desoxycholate, which removed some inactive protein, then extracting the residue with the higher concentrations of desoxycholate. Sodium azide, sodium cyanide, and carbon monoxide inhibit the extracted oxidase in the dark, the carbon monoxide inhibition being reversed in light.

Treatment of cytochrome oxidase with ultrasonic waves as applied by Haas (130) did not bring cytochrome oxidase into solution according to Keilin & Hartree (131) nor raise the activity of the preparation. Haas' claim (130) that the oxidase may be split into two components is disputed (131), since one component was successfully replaced by plasma proteins or by small amounts of denatured globin.

The oxidation of ferrocytochrome-*c* by cytochrome oxidase from rat heart and brain and from frog skeletal muscle was followed

spectrophotometrically by Horecker & Stannard (132), both cyanide and azide being able to inhibit oxidation completely. Complexes form between the oxidase and the undissociated acids, hydrozoic acid and hydrocyanic acid, while the combinations of ferricytochrome-*c*, ferrihemoglobin, and ferrimyoglobin occur with the corresponding ions. The dissociation constants for the enzyme-inhibitor complex was 7×10^{-7} for hydrocyanic acid.

Cytochromes.—A comparison of methods of preparation and of the properties of cytochrome-*c* by Keilin & Hartree (131) indicated that material prepared by Haas' method (133) was less active and inferior to material prepared by their method (134).

Ferricytochrome-*c* and azide ion reversibly form a complex containing one mole of azide per mole of cytochrome as found spectrographically by Stannard & Horecker (135). Although cyanide forms a stable complex of dissociation constant 2×10^{-6} , the rate of combination is such that only half the cytochrome is combined after 60 min. in 0.001 *M* potassium cyanide; the constant for azide is much higher, 0.15, but equilibrium is reached within 0.5 min. The fraction of ferricytochrome combined at pH 7.4 in 0.001 *M* inhibitor at 25°C. is 87 per cent with cyanide and practically none with azide.

Intravenous administration of cytochrome-*c* has been reported by Rabinovitch, Elliott & McEachern (136) to be apparently harmless but of no noticeable benefit to cases of neuromuscular disease in man. The diminution of readily hydrolyzable phosphate in the rat kidney and heart caused by anoxia can be prevented by previous injection of cytochrome-*c* according to Miller, Anderson & Dorfman (137). Anoxia was found by Drabkin (138) to be without apparent effect in the rat following partial hepatectomy either upon liver regeneration or upon the metabolism of cytochrome-*c*. Cytochrome-*c* injections did not protect the normal rats of Christensen & Clinton (139) against the effects of anoxia.

The principal iron porphyrin-containing pigment of the diphtheria bacillus grown on media containing an excess of iron is apparently cytochrome-*b*, according to the spectrophotometric data of Pappenheimer & Hendee (140), a conclusion which substantiates the early work of Fujita & Kodama in 1934. Since the apparent increase in cytochrome-*b* in the cells was found to be equivalent to the iron taken up from the medium and to the toxin which failed to be excreted by the cells into the medium, it is suggested (140)

that the protein of the diphtheria toxin forms a complex with cytochrome-*b* within the cells. Rawlinson & Hale (141) confirm the presence of cytochrome-*b* in the bacterial pigment.

Correlation between the hematin concentration of root nodules and nitrogen fixation was indicated in the comparative experiments of Virtanen, Erkama & Linkola (142) on a large number of strains of nitrifying bacteria. Extracts of the red nodules showed strong absorption maxima at 555 and 525 $m\mu$, which disappeared after the nodules turned green; at this time leghemoglobin, a heme-protein of legumes, was no longer found and the nitrogen fixation had ceased.

The mode of action of the hemoproteins in relation to their chemical constitution is the subject of a review by Theorell (143) and the heme proteins are discussed in an extensive review by Wyman (144). Continuing the series of investigations on metalloporphyrins, Shack & Clark (145) studied the cycles of changes in systems containing heme in buffers alone and with pyridine or cyanide by spectrophotometric and oxidation-reduction potential techniques and developed the theoretical and mathematical relationships involved.

Catalase.—An intermediate compound of catalase with hydrogen peroxide was detected by Chance (146, 147) by his previously developed microspectrophotometric adaptation of Hartridge & Roughton's and Millikan's apparatus for kinetic studies; three of the four hematin-iron groups of catalase are still free and capable of combining with cyanide, so that one molecule of enzyme is combined with only one molecule of substrate. The addition of more than one hydrogen peroxide to catalase is believed to result in an as yet undetected complex which then rapidly decomposes. Alcohols are oxidized by the catalase-hydrogen peroxide compound, as with the peroxidase-hydrogen peroxide complex, which probably explains the "coupled oxidations" of alcohols described by Keilin & Hartree (148).

Alkyl hydroperoxides combine with all four of the catalase hematin-iron groups (146), forming primarily greenish compounds and secondarily red compounds analogous to the horse-radish peroxidase-hydrogen peroxide compounds of Theorell (149). The alkyl hydroperoxide catalase compounds also react with alcohols, behaving like peroxidases.

Higher concentrations of enzyme are recommended by Bonnichsen, Chance & Theorell (150) in the measurement of catalase activity; the velocity constant for the reaction of enzyme with substrate is then large and nearly constant, the partial inactivations otherwise occurring during measurement being minimized.

A review of catalase experimentation along the foregoing lines is presented by Theorell (151), who concludes that the peroxidative action of catalase may explain why the catalase concentration of many tissues is higher than would be necessary for merely hydrogen peroxide decomposition, and how the waste of energy is avoided that would result from the decomposition of hydrogen peroxide into water and oxygen.

Tyrosinase.—The enzymatic oxidation of 3,4-dihydroxyphenylalanine was followed spectrophotometrically by Mason (152), who observed three chromophoric phases, the formation of a red pigment, then an intermediate purple pigment, and finally melanin; absorption maxima of 305 and 475 $m\mu$, 300 and 540, and general absorption, respectively. The first phase appears to be the formation of 2-carboxy-2,3-dihydroindole-5,6-quinone, which then forms 5,6-dihydroxyindole. The latter, when enzymatically oxidized, gives a spectrum like that of the purple second phase. Since 5,6-dihydroxyindole is readily oxidized in presence of tyrosinase to indole-5,6-quinone, which possesses several reactive groups, polymerization of this compound is considered a possible pathway to melanin.

The oxidation of some substituted catechols and phenols by tyrosinase was measured by Cushing (153) and the number of oxygen atoms consumed per mole determined. That 4-substituted catechols result from addition to an *o*-quinone is well known, and Dawson & Nelson (154) believe that water adds in a similar manner to form 4-hydroxy-1,2-hydroquinone which is then further oxidized enzymatically, using a second oxygen atom, to give hydroxyquinone. Cushing showed that with ortho-directing groups (Cl, CH₃) in the 4-position of catechol the number of oxygen atoms consumed is two, the same as for catechol, the oxidation being directed to the 5-position which is ortho- to 4 and para- to the quinone=CO at the 2-position. Meta-directing groups (—CHO, —COOH, —SO₃H) in the 4-position permit only one oxygen to be used, directing into 2, which is already oxidized, and into 6 which

is not para- to $\text{a}=\text{CO}$, an apparent requirement. With di-substituted catechols having an ortho-directing group in the 4-position and with 5 filled, only one oxygen atom is used. Hydrogen peroxide is apparently not produced in any case. With substituted phenols having an ortho-directing group (Cl) in the 4-position, three atoms of oxygen are consumed per mole, while no reaction takes place with meta-directing groups ($-\text{CHO}$, $-\text{COOH}$); the results of prior work are consistent with these conclusions.

OXIDATIONS AND REDUCTIONS OF CYCLITOLS

The oxidation of isomers of the inositol group and related cyclitols by *Acetobacter suboxidans* was found by Magasanik & Chargaff (155) to occur only at hydroxyls situated in a polar plane in the molecule. *Epi*-inositol gave only *l-epi*-inosose, confirming Posternak (156), while *l*- and *d*-inositol gave products from which the osazones of α -diketones were isolated but the diketones themselves were not prepared; oxygen absorption results were in agreement. Scyllitol and the methyl ethers, quebrachitol and pinitol, were not oxidized, but *d*-quercitol consumed two oxygen atoms per mole (155). *Scyllo*-inosose was the only product of *meso*-inositol oxidation found by Carter and co-workers (157) confirming Posternak and Kluyver & Boezaardt, but in disagreement with Dunning *et al.* [cited in (157)] who claimed diketone production based on osazone formation. Since di-ketones can be produced from mono-ketones by the phenylhydrazine used for isolation, the oxygen consumption methods seem more valid for establishing the extent of oxidation. The results (155, 157) are in agreement with the configurational structures of inositols developed previously by Posternak (158). Incomplete oxidation of *d*-inositol produced *d*-inosose which was isolated by Magasanik & Chargaff (159). Scyllitol and *meso*-inositol have been prepared from hexahydroxybenzene by Anderson & Wallis (160) using hydrogen and the catalyst, Raney nickel. A monograph by Fluery & Balatre reviews the chemistry and biochemistry of inositols (161).

Three inosomines, monoamino analogues of inositol, have been prepared by Carter and co-workers (162), two by reduction of *scyllo*-inosose phenylhydrazine or oxime, and one of the two expected from the reduction of *dl-epi*-inosose phenylhydrazine. The bacterial oxidation of 1,2,3-trihydroxycyclohexane has been studied by Posternak (163) and its configuration deduced.

THIOL GROUPS AND ENZYMES

A review by Peters (164) outlines the development and theoretical significance of 2,3-dimercapto-propanol, (BAL), its relation to the pyruvic-oxidase system and to the arsenicals, and its usefulness in the treatment of the lesions caused by the arsenical vesicants. The therapeutic applications of BAL to the disturbances caused by the toxic action of arsenic, gold, and other metallic derivatives is reviewed by Thompson (165). Many more of the investigations carried on during the war, whose publication was then restricted, have been released this year and greatly increase our knowledge of the types of compounds which inhibit enzymes.

Organic arsenicals, phenylarsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide (mapharside), *p*-acetaminophenylarsenoxide, and *m*-acetamido-*p*-carboxyphenyl-arsenoxide, and the reduced forms of atoxyl and tryparsenamide, inhibit the typical thiol enzymes, urease, pyruvic oxidase, succinic dehydrogenase, and choline esterase, according to Gordon & Quastel (166), the toxicity being diminished or eliminated by the addition of glutathione or cysteine. The pentavalent arsenic compounds may be reduced by the proper quantity of thiols to the toxic trivalent state. Choline dehydrogenase is established as a thiol enzyme (166).

Lachrymators, such as chloracetophenone, brombenzyl cyanide, chlorpicrin, and ethyl iodoacetate, have been shown by Mackworth (167) to inhibit irreversibly enzymes in a manner similar to iodoacetic acid. Muscle respiration and the respiration and fermentation of glucose by yeast are also inhibited. A considerable excess of the simple thiols is required to protect against these inhibitions. The action of chlorpicrin upon yeast is reversible. The inhibitor is apparently destroyed by the yeast so that simple washing restores activity.

Using crystalline yeast hexokinase, Bailey & Webb (168) confirmed the work of van Heyningen (169) with crude extracts, which indicated that the enzyme is inhibited by thiol reagents, and included further the lachrymators and vesicants. When mustard gas, $(\text{ClCH}_2\text{CH}_2)_2\text{S}$, in dilute solution containing radioactive sulfur acted on hexokinase, it was estimated that 6 to 7 molecules per enzyme molecule were necessary for complete inhibition. When the enzyme was treated with the liquid vesicant, Herriott, Anson & Northrop (170) found 30 to 35 molecules were taken up. The latter figure is believed (170) to be due to a more general attack

upon the enzyme, while 6 to 7 is more of the order of the number of active centers.

Certain dithio-compounds, N:N-diethyl-dithiocarbamate and N:N-di(2-hydroxyethyl)-dithiocarbamate, instead of protecting the pyruvic-oxidase against mustard gas, were found by Holiday and co-workers (171) to increase or "potentiate" the toxicity. Compounds of mustard gas $(\text{ClCH}_2\text{CH}_2)_2\text{S}$ with one or two molecules of $(\text{C}_2\text{H}_5)_2\text{N}\cdot\text{C}=\text{S}\cdot\text{SH}$ or of $(\text{HOC}_2\text{H}_4)_2\text{N}\cdot\text{C}=\text{S}\cdot\text{SH}$ were prepared by Peters & Wakelin (172) and tested for toxicity upon pigeon brain brei respiration. Some evidence is presented to indicate that the increased toxicity may be due to the $=\text{N}\cdot(\text{C}=\text{S})\cdot\text{S}$ -group, since chlorine-free compounds were also toxic.

Nitrogen mustards, $(\text{ClCH}_2\text{CH}_2)_2\text{N}$, or the less halogenated compounds, such as $(\text{ClCH}_2\text{CH}_2)_2\text{N}(\text{CH}_3)$, after removal of hydrogen chloride, did not inhibit the activity of the thiol enzymes, succinic oxidase, D-amino acid oxidase, and phosphoglyceraldehyde dehydrogenase at the concentrations usually causing inhibition, although they reacted generally with thiol groups. Further, Barron, Bartlett & Miller (173) found that they did inhibit choline oxidase, choline esterase, and betaine oxidase, in relatively very low concentrations; they concluded inhibition was due to structural similarity of the ethylenimmonium derivatives to choline and attachment to the enzyme where combination with choline takes place. For those enzymes that are inhibited, phosphokinases, pyruvic oxidase, etc., interaction of the reactive halogen through the thiol or other reactive groups of the enzyme is assumed. Barron *et al.* (174) extended the experiments to include tissue slices and administration of the substances to rats.

The function of the thiol groups in "sulfhydryl enzymes" may be a direct participation in the activity or they may be located at the "active centers" where combination of enzyme or substrate takes place, as related by Potter & DuBois (175). If the sole function of the thiol group of the enzyme is to provide a locus for combination with the substrate, then Singer (176) believes that the inhibition of the sulfhydryl enzyme by a specific thiol reagent should be the same, regardless of the substrate used, provided the enzyme is the limiting factor in the activity determination. In agreement with this, his experiments (176) show the inhibition of D-amino acid oxidase to be independent of the nature of the substrate used. However, with wheat germ lipase, the inhibition by

the sulfhydryl reagents, *p*-chlormercuribenzoate and *o*-iodosobenzoate, varied with the particular substrate used in apparent relation to the molecular size of the substrate, the larger the substrate the greater the inhibition. This suggests steric interference by the inhibitor, attached through the thiol or removing thiol by oxidation, to the approach of the larger molecules to the "active center" neighboring the thiol group involved. A corollary to this theory would be that the molecular size of the inhibitor would similarly be a factor in interfering with the approach of a substrate to the "active center."

Oxidation of the thiol groups of enzymes rather than the production of a toxic organic complex (177) is considered the basis of the bactericidal action of chlorine by Knox, Stumpf & Green (178), who showed that the aldolase of *Escherichia coli* is sufficiently sensitive to chlorine oxidation to explain the bactericidal effect. The inhibition of carboxylase by quinones is believed by Kuhn & Beinert (179) to be due to oxidation of one or more thiol groups.

OXIDATION-REDUCTION POTENTIALS AND MECHANISMS

The stability of semiquinone radicals has been shown by Granick & Michaelis (180) to depend on the ease of obtaining the required proton for attachment to the bridging N of thiazine types. The system of which thionol is the reductant was measured and the points of intersection of the three principal E'_0 -pH curves (quinone—semiquinone, semiquinone—hydroquinone, quinone—hydroquinone) for this and other thiazine systems were compared. With amino-thiazines, where the additional amino groups compete with the bridging N for the proton higher acidities (such as pH of -5) are required for stable semiquinones than for thional (pH of +2).

Four-valent change systems are considered possible by Preisler, Berger & Hill (181, 182) for compounds having two two-valent steps from oxidant to reductant. The hexahydroxybenzene—tetrahydroxyquinone system overlaps or merges with the tetrahydroxyquinone—rhodizonic acid system from about pH 5 to 7, making possible significant concentrations of the hexahydroxybenzene—rhodizonic acid system with a four equivalent change. The last system may be of importance when these substances are used

in catalytic reactions involving more than two equivalents. Schwarzenbach & Suter (183) had reported similar results. The compounds mentioned are found among the chemical and bacterial oxidation products of inositol.

Many investigators [cited in (184, 185)] have attempted to measure the potentials of simple thiol—disulfide systems, such as cysteine—cystine and thioglycollic acid—dithiodiglycollic acid, either directly by electrometric methods or indirectly by indicators, in every case with unsatisfying results. The latest attempt, by Rykhan & Schmidt (184), reported extensive electrometric measurements on a number of thiol—disulfide systems made with the aid of iodide as catalyst, with the results apparently conforming to the theoretical equations. In view of the disagreement with previous work and the difficulties in measuring such systems, this subject should be reinvestigated and the results confirmed before application to problems of thiol—disulfide chemistry.

A thiol—disulfide system of the type, $2\text{RSH} = \text{RSSR} + 2e + 2\text{H}^+$, has been measured successfully without any additional catalyst by the usual potentiometric methods by Preisler & Berger (185). Thiourea—formamidine disulfide gives stable reproducible potentials with $E_0' = +0.420$ volt at pH 0, the potential varying accurately with the equation $E = E_0' + 0.03 \log (\text{RSSR})/(\text{RSH})^2$. The E_0' -pH curve has a slope of 0.00 from pH 0 to 4, showing that the oxidant is $++$ charged in this pH range. An important consideration in the measurement or use of such systems is the $+0.03$ volt change in potential on tenfold dilution of a fixed ratio of reactants, particularly for biological systems where great changes in concentration may take place. Substituted thioureas were studied by Preisler (186) and found to have the same characteristics as thiourea, the highest E_0' at pH 0 was $+0.511$ for NNN'-trimethyl thiourea.

Thiol—disulfide systems such as $\text{R}(-\text{SH})_2 = \text{R}(-\text{SS}-) + 2e + 2\text{H}^+$, where the oxidant is a ring or cyclic disulfide, have been measured electrometrically by Preisler & Bateman (187) and Preisler (186) and satisfy equations for two-equivalent change systems. The dithiobiuret—3,5-diimino-1,2,4-dithiazolidine system (187) gave stable reproducible potentials, with $E_0' = +0.251$ volt at pH 0 and slope of E_0' -pH curve of -0.03 volt from pH 0 to 5, showing that one proton is involved in the reaction in this pH range. Reductants of similar systems having two thiourea type (thiocarbaminy1-

groups attached to a single molecule were prepared (186) from diamino aliphatic hydrocarbons by reaction with ethyl isothiocyanate; the largest ring structure oxidant studied contained 2 sulfur, 6 carbon, and 2 nitrogen atoms. The potentials of these systems were of the same order as that of the thiourea—formamidine disulfide system, indicating that the cyclic disulfide is formed with about the same ease as the disulfide between two molecules, an important consideration for certain problems relating to proteins or enzymes. There is essentially no change in potential for diluting a fixed ratio of oxidant to reductant for systems with the ring disulfide oxidant.

A review of enzymatic oxidations and reductions by LuValle & Goddard (188) emphasizes mathematical and physicochemical considerations for the processes involved. Semiquinones are believed to be involved (188) in many enzymatic reactions; either the donor or the acceptor may combine with the enzyme and undergo an univalent oxidation-reduction, then the enzyme-semiquinone complex will react with the other reactant to form the final product. The rates of formation of semiquinones is considered an important factor in the rate of autoxidation of *p*-phenylenediamine and its derivatives by LuValle, Glass & Weissberger (189); the biological implications of such mechanisms are discussed by LuValle (190), who contrasts the semiquinone mechanism with the chain mechanism involving peroxide formation.

A comprehensive book by Clark (191) covers the many phases of physical chemistry in its application to problems of biology and medicine and includes several chapters on oxidations and reductions and related topics.

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PROTEOLYTIC ENZYMES¹

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EXOPEPTIDASES

Knowledge of the exopeptidases has now developed to the point where some generalizations have become apparent which are likely to have a marked influence on the future work in this field. It is now clear that the peptidases of intestinal mucosa, the classical source of these enzymes, represent an extremely complex mixture of enzymes of exceedingly diverse specificity (1). The term erepsin then becomes a misleading one in so far as it is used to describe a single enzyme or mixture of a few enzymes. Secondly, the same or similar enzymes have now been found in many types of animal tissues, such as spleen, liver, kidney (2), skin, lung, serum (3), in various types of muscle tissue (4), and in thymus (5). Related peptidases have also been studied in bacteria, fungi, and green plants (6). Undoubtedly, these enzymes will also be found elsewhere. The widespread distribution of these enzymes raises the question as to the function of these peptidases in the various tissues.

The work of Johnson & Berger (6) demonstrated that the rate of hydrolysis of certain peptides by tissue extracts is markedly enhanced by the presence of metal ions. It is now evident that almost all of the exopeptidases are true metal-protein compounds (7). Since the metals are rather easily removed, it is necessary that assays of these enzymes be performed under conditions where their full activity is manifested. Even dilution may effect an apparent loss of enzyme activity unless the concentration of metal ions is kept constant. Failure to determine the optimal conditions for the assay of crude tissue extracts throws some doubt on the absolute significance of many investigations in which quantitative comparisons have been made of the relative distribution of peptidase activities in various cells, tissues, and organs.

¹ This review deals mainly with the period from December 1, 1946 to November 1, 1948.

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Another factor of theoretical as well as practical importance is the observation that the reactivation of certain peptidases by a specific metal is a time reaction. This has been demonstrated for leucine aminopeptidase (8), prolidase (4), and glycyl-L-leucine dipeptidase (9). Unless the activation of the enzyme is complete, as shown by regular reaction kinetics, extremely misleading assays of enzyme activity will be obtained. The slow activation of the specific protein by the metal ion indicates that some form of coordination is taking place rather than a simple ionic reaction. This, together with evidence from studies on glycyglycine dipeptidase, has led to the suggestion (10, 11) that, in the peptidases, the role of the metal is to act as a bridge in forming the enzyme-substrate complex. This will be discussed further in the section on dipeptidases.

Carboxypeptidases.—Our knowledge of the specificity of crystalline pancreatic carboxypeptidase has been extended by a study of the hydrolysis of carbobenzoxy derivatives of tryptophane peptides (12). Carbobenzoxyglycyl-L-tryptophane is hydrolyzed almost as rapidly as the corresponding substrates containing aromatic nuclei, namely, carbobenzoxyglycyl-L-phenylalanine and carbobenzoxyglycyl-L-tyrosine. The hydrolysis of tryptophane derivatives follows zero order kinetics under the same conditions in which that of the other substrates follows first order kinetics (13). It is of interest that the specificity relations elaborated by Bergmann & Fruton (14) for this enzyme seem to hold regardless of the particular type of reaction kinetics. Thus, the ratio of the zero order velocity constants for carbobenzoxy-L-tryptophyl-L-alanine and carbobenzoxy-L-tryptophylglycine is the same as the ratio of the first order constants for carbobenzoxyglycyl-L-alanine and carbobenzoxyglycylglycine. The hydrolysis of carbobenzoxy-L-tryptophylglycine is about three times as fast as that of carbobenzoxyglycylglycine (12). However, the splitting of carbobenzoxyglycyl-L-tryptophane is nearly 2,000 times more rapid than that of carbobenzoxyglycylglycine. This is consistent with earlier findings that the nature of the residue possessing the free carboxyl group is the main determinant of sensitivity of the substrate to the enzyme (13, 14).

Of great interest is the observation (15) that carboxypeptidase, like trypsin and chymotrypsin (see below), possesses an esterase activity. Carboxypeptidase hydrolyzes with zero order kinetics

hippuryl- β -phenyl-lactic acid; this compound is the ester analogue of the most sensitive peptide substrate for the enzyme. Neurath, Elkins & Kaufman (16) have found, in contradistinction to previous reports, that the rate of hydrolysis of carbobenzoxyglycyl-L-phenylalanine is independent of the presence of the D-form of this compound. However, D-phenylalanine and other D-amino acids act as competitive inhibitors of the enzyme (17). It was also found that carboxypeptidase action does not follow simple first order kinetics. The increase of the approximate first order constants was shown to be related to the change in equilibrium between the free enzyme and the enzyme-substrate complex with varying substrate concentration. A velocity constant which is independent of both enzyme and substrate concentration and characteristic of the system was obtained by integration of the Michaelis-Menten equation.

Some physical studies have been made on carboxypeptidase. Putnam & Neurath (18) have found that six-times crystallized carboxypeptidase is homogeneous electrophoretically below pH 8.5 and possesses an isoelectric point near pH 6.0 when measured in univalent buffers of 0.2 ionic strength. The average diffusion constant, D_w , 25° is 9.4×10^{-7} sq. cm. per second. From this and viscosity measurements, they calculated a dissymmetry constant $f/f_0 = 1.16$ and a molecular weight of 31,600.

Smith & Hanson (19) have observed that carboxypeptidase is strongly inhibited by sulfide and cyanide. Significant amounts of magnesium were detected in the residual ash of the five-times crystallized enzyme. The enzyme appears to be a metal-protein like other exopeptidases. It differs from these only in so far as the metal is much more firmly bound to the protein.

It has previously been pointed out that the conjugases which act on folic acid conjugates apparently possess the specificity of carboxypeptidases (20). Mims, Swendseid & Bird (21) found that the conjugases from hog kidney, hog intestine, rat liver, and human leucocytes are inhibited by nucleic acid. They have also reported the presence of an active sulfhydryl group in conjugase from hog kidney.

Kazenko & Laskowski (22) have partially purified the conjugase of chicken pancreas. They could not detect any hydrolysis of an initial glutamic acid residue linked either through the α -carboxyl or through the γ -carboxyl in glutathione. Their enzyme hydrolyzed

the terminal glutamic acid residue from *p*-aminobenzoyl- γ -glutamylglutamic acid, synthetic pteroyl triglutamate, and natural fermentation factor. Only one equivalent of glutamic acid was recovered; this is assumed to be due to the inhibition of the conjugase by free glutamic acid. They suggest that the conjugase of chicken pancreas should be classified as a γ -glutamic acid carboxypeptidase requiring at least two terminal glutamic acid molecules in the peptide chain. Earlier work on the conjugases and on folic acid conjugates is reviewed by Jukes & Stokstad (23).

Tripeptidase.—It has been reported previously (1) that a substantial portion of the activity of extracts of intestinal mucosa towards tripeptides such as L-leucylglycylglycine and diglycylglycine is due to an enzyme which does not appear to require metal activation. The partially purified enzyme has little action on dipeptides and tetrapeptides. Enzymes of related specificity have been identified in skin (3) and in muscle (4).

Fruton, Smith & Driscoll (5) now describe a similar enzyme from calf thymus which they call lymphopeptidase. They could not detect any metal activation nor any inhibition by the known poisons of metal-enzymes. The enzyme showed no significant hydrolytic action on dipeptides and tetrapeptides. However, it acted on a wide variety of synthetic tripeptides in which both terminal amino and carboxyl groups were free by hydrolyzing the peptide bond adjacent to the free amino group. The kinetics of hydrolysis followed those of a zero order reaction. The enzyme does not hydrolyze D-leucylglycylglycine, or glycyl-D-leucylglycine but hydrolyzes the corresponding L-compounds quite rapidly. Even more interesting is their observation that glycylglycyl-D-leucine is hydrolyzed at less than one-fourth the rate of the corresponding L-compound. They suggest that this enzyme be classified as an aminoexotripeptidase.

A further attempt has been made (24) to relate the aminopeptidase (substrate: L-alanylglycylglycine) isolated from hog stomach to the "intrinsic factor" of Castle. It has been found that beef muscle predigested with pepsin and then treated with the purified aminopeptidase yields a factor which appears to be capable of inducing remission in pernicious anemia patients.

Leucine aminopeptidase.—Leucine aminopeptidase has been found in muscle, heart, and uterus (4) in addition to other sources described earlier (6). Although the enzyme may be activated by

either magnesium or manganous ions, it is found in tissues as a magnesium-enzyme as judged by the strong inhibition produced by citrate. The enzyme has been regarded as the prototype of an aminopeptidase because of its strong action on L-leucinamide. It has now been found (25) that the enzyme also acts rapidly on such compounds as glycyl-L-leucinamide and L-glutamyl-L-leucinamide and possesses a slower action on diglycyl-DL-leucylglycine. The hydrolytic action is on the linkage of the leucine carbonyl. Thus, the free amino group which is required by the enzyme can be present at least one peptide bond removed from the sensitive linkage. The enzyme does not possess any endopeptidase activity as judged by its failure to split compounds like carbobenzoxyglycyl-L-leucinamide and carbobenzoxydiglycyl-L-leucinamide.

The older concept of an aminopeptidase which hydrolyzes the peptide bonds adjacent to a free amino group and which acts on polypeptides regardless of their chain length is now subject to considerable doubt. The tripeptidase discussed in the previous section has little or no action on tetrapeptides although its specificity is essentially that of an aminopeptidase. Moreover, it is quite unspecific towards the residue which bears the free amino group. On the other hand, leucine aminopeptidase appears to be specific towards leucine compounds. Its only known action is on peptide bonds which involve the carbonyl of leucine. The newly discovered action of leucine aminopeptidase on compounds like glycyl-L-leucinamide shows that not only do the various aminopeptidases possess quite different specificities, but also that the aminopeptidase action of this enzyme is not necessarily restricted to the peptide bond adjacent to the free amino group. It is apparent from these recent studies that the classical definition of an aminopeptidase must be modified. However, in the opinion of this reviewer, it would be premature to attempt any substitute classification at this time.

There has been found, in extracts of human uterus, in addition to the leucine aminopeptidase, another enzyme which hydrolyzes L-leucylglycine (4). This enzyme can be distinguished from the leucine aminopeptidase since it does not appear to be metal activated and since it has little or no action on L-leucinamide.

Dipeptidases.—Until recent years, it was generally assumed that the hydrolysis of all simple dipeptides was due to a single dipeptidase. In most cases, studies were performed on L-leucylglycine or L-alanyl-glycine. Since these two substrates have been shown to

be hydrolyzed by different enzymes, and since the hydrolysis of L-leucylglycine by most tissue extracts has been shown to be due to an aminopeptidase, the existence of a dipeptidase which is capable of hydrolyzing many kinds of dipeptides has become doubtful (1). The studies of Bergmann and co-workers (26, 27) have amply demonstrated that proteolytic enzymes act only on peptide bonds involving specific amino acid residues in addition to a requirement for the presence or absence of amino and carboxyl groups. Certain substrate-specific dipeptidases have now been described.

Glycylglycine is hydrolyzed by a specific dipeptidase which is strongly activated by cobaltous ion and, in some cases, to a lesser extent by manganous ion (10). Hydrolysis may be prevented by substitution of the amino group (benzoylglycylglycine), the carboxyl group (glycylglycinamide), or both (carbobenzoxyglycylglycinamide). The enzyme hydrolyzes sarcosylglycine at about one-twelfth the rate of glycylglycine, but N-dimethylglycylglycine is resistant to hydrolysis, showing that at least one free hydrogen must be present on the terminal amino group (11). Diglycylglycine and glycylsarcosine are also unaffected by the dipeptidase. The enzyme is widely distributed in mammalian tissues, and, in most instances, is extremely labile. This has served to differentiate the dipeptidase from more stable enzymes which act on other simple dipeptides. In contrast, the specific dipeptidase of human uterus is extremely stable and has been used for the study of the kinetics of this enzyme. At a substrate concentration of 0.05 *M*, the hydrolysis follows the kinetics of a zero order reaction. Its optimal action is at pH 7.6 and the dissociation constant of the enzyme-metal compound is $2.8 \times 10^{-5} M$.

When glycylglycine is incubated with cobaltous ion at pH 7.6 to 8.0, a pink color develops which is much stronger than that given by cobaltous ion alone, or by cobaltous ion and glycine or diglycylglycine. A parallelism has been found between the ability of cobaltous ion to coordinate with potential substrates and the susceptibility of the compound to enzymatic action. Smith (11) has suggested that the role of the metal in this and other peptidases is to act as a bridge in forming the enzyme-substrate compound. Thus, the specificity of the enzyme would depend not only on the protein, but also on the ability of the metal ion to form a coordination complex with the substrate. This is emphasized by the demon-

stration that the coordination of metals and peptides may be extremely specific. For example L-leucylglycine coordinates much more strongly with cobaltous ion than does the isomeric peptide, glycyl-L-leucine. This concept of metal coordination compounds involving enzyme and substrate is the first attempt to explain the role of metal ions in proteolytic reactions. Hellerman and his co-workers (28) had proposed earlier a similar role of metal ions in the action of arginase.

The enzymes of various mammalian tissues which act on glycyl-L-leucine also possess dipeptidase specificity since carbobenzoxyglycyl-L-leucine, carbobenzoxyglycyl-L-leucinamide, and glycyl-L-leucinamide are not attacked by these enzymes (9). On the other hand, the methylated peptide, sarcosyl-L-leucine, is split slowly. The metal ions required for activation appear to be highly specific for the glycyl-L-leucine dipeptidases of different tissues. The enzymes of rabbit muscle and hog intestinal mucosa are strongly activated by manganous ion, those of human uterus and rat muscle by zinc ion. In all cases, maximal activity is obtained with phosphate buffer apparently because of the removal of calcium which acts as an inhibitor. With the activating metal and phosphate the hydrolysis of glycyl-L-leucine is a first order reaction and the rate is proportional to the enzyme concentration over a wide range. When the glycyl-L-leucine dipeptidase of hog intestinal mucosa is precipitated with ammonium sulfate and dialyzed, the activation of the protein by manganous ion becomes a time reaction. The activity of the enzyme at different manganous ion concentrations shows a typical mass law relationship with an apparent dissociation constant of the manganous ion protein complex of $1.8 \times 10^{-4} M$.

Praetorius (29) has studied the enzymes of thymus which act on DL-alanylglycine; these are assumed to be dipeptidases. He differentiates between a labile enzyme which appears instantly in the dispersion medium and a less labile fraction which is slowly extracted from the cells by salt solution. Both enzymes are stabilized by cold glycerol. The more labile enzyme may originate from the reticulum cells, and the other from lymphocytes although the evidence is regarded as not conclusive.

Pope & Anfinsen (30) have estimated the alanylglycine-splitting activity of the central nervous system of the rat. Greater rates of hydrolysis are found in extracts from the cerebrum or cerebellum than in preparations from brain stem and spinal cord.

The amount of activity appears to be related to the number of nerve cell bodies present in the tissue sample.

Prolidase.—This enzyme which is apparently specific for peptide bonds which involve the imino nitrogen of proline and hydroxyproline is widely distributed in animal tissues (1, 3, 4). These enzymes are homospecific as judged by the constant ratio of activity observed for the hydrolysis of glycyl-L-proline and glycyl-hydroxy-L-proline (4). All of the prolidases appear to be specifically activated by manganous ion. The activation of the purified enzymes by manganous ion is a time reaction (4). The prolidase of rabbit muscle possesses its maximal activity between pH 7.5 and 8.2.

Dehydropeptidases.—The work on this group of enzymes is the subject of a recent detailed review by Greenstein (31) which provides an excellent coverage of the field. In view of this, only a brief summary of some of the recent publications will be given here.

Two dehydropeptidases are differentiated (31), dehydropeptidase I which is present in all tissues and for which glycyldehydroalanine is a suitable substrate, and dehydropeptidase II which is located in only a few tissues and for which chloroacetyldehydroalanine is the substrate. The two enzymes exhibit different pH optima. In both cases, ammonia and pyruvate are formed. The reactions may be followed by chemical estimation of the reaction products, or spectrophotometrically since the dehydropeptides show strong ultraviolet absorption while the products do not (32, 33).

Yudkin & Fruton (33) have partially purified the dehydropeptidase of rat kidney which acts on glycyldehydrophenylalanine. This enzyme is inhibited by sulfide, cyanide, and cysteine. Prolonged dialysis causes a diminution of activity which can be specifically restored by zinc salts (37). It appears that this enzyme, like many other exopeptidases, is a metal-protein.

Greenstein and his co-workers have devoted considerable attention to the possible natural occurrence of dehydropeptides. Some findings have suggested four possible ways in which they may originate: from the desulfuration of certain cystine peptides (34), by the action of D-amino acid oxidase on D-peptides such as glycyl-D-alanine (31), by the degradation of compounds like α - α -diglycylaminopropionic acid to glycynamide and the dehydropeptide (35),

and by the condensation of glutamine or asparagine with α -keto acids to form the dehydropeptide (36).

Hydrolysis of β -alanine peptides.—On the basis of work reported some years ago by Abderhalden *et al.* (38, 39) the opinion has been widely held that peptides containing β -amino acids are not hydrolyzed by proteolytic enzymes. In contrast to this, it has been found by Hanson & Smith (40) that various peptidases will hydrolyze synthetic peptides containing β -alanine provided the compounds are related to the usual substrates for the different enzymes. Leucine aminopeptidase of hog intestinal mucosa hydrolyzes L-leucyl- β -alanine at almost the same rate as L-leucinamide and L-leucylglycine. Crude aminopeptidase preparations of human uterus and rat muscle also hydrolyze L-leucyl- β -alanine. With these aminopeptidases it would appear that the main specificity of the enzyme is directed towards the leucine residue and that the moiety attached to the leucine carbonyl has little effect in determining the sensitivity of the substrate.

Crystalline pancreatic carboxypeptidase hydrolyzes carbobenzyloxylglycylamino acids about 800 to 1,600 times faster than the corresponding carbobenzoxy- β -alanyl amino acids as observed with the leucine and phenylalanine compounds. Although the nature of the residue bearing the acyl group has generally been regarded as being of secondary importance in determining the sensitivity of the substrates for this enzyme it is clear that this is not the case since the intercalation of a CH_2 group has such a marked influence on the rate of hydrolysis.

With enzymes which require a free amino group adjacent to the sensitive peptide bond such as prolidase, glycyl-L-leucine dipeptidase, and glycylglycine dipeptidase, increasing the distance from the free amino group to the peptide bond decreases the sensitivity of the substrate quite markedly. This is illustrated by the purified prolidases of hog intestinal mucosa and of rabbit muscle which hydrolyze glycyl-L-proline about 200 to 300 times more rapidly than β -alanyl-L-proline. Moreover, the hydrolysis of glycyl-L-proline is inhibited about 50 per cent in the presence of an equimolar concentration of the β -alanyl compound.

Hydrolysis of glutathione.—Binkley & Nakamura (41) have found that an enzyme, limited to the kidney in the rat, liberates cysteinylglycine from glutathione by the hydrolysis of the γ -

peptide linkage. This enzyme is maximally active at about pH 7.5 to 8.0 and does not appear to require metal activation except at high substrate concentrations where magnesium seems to have some effect. The cleavage of cysteinylglycine is performed by a second enzyme found in liver, muscle, and other tissues as well as kidney.

Protaminase.—Portis & Altman (42) find that about one half of the amino nitrogen of salmine is liberated by crystalline trypsin or crystalline chymotrypsin. However, crystalline pepsin has no action on the protamine. Crude preparations of trypsin which contain protaminase liberate free arginine from salmine as estimated by arginase action; this is in contrast to the action of crystalline trypsin and chymotrypsin where no free arginine could be detected. It may be inferred from these data that "protaminase" is probably an exopeptidase which acts on the arginine peptides liberated by tryptic action. The reviewer has observed that salmine is attacked by crystalline carboxypeptidase. The possible identity of protaminase and carboxypeptidase is being studied.

ENDOPEPTIDASES

A revised edition of the book on *Crystalline Enzymes* by Northrop *et al.* (43) is now available. This includes all of the work by the Rockefeller group on proteolytic and other enzymes.

Pepsin.—The older work on the occurrence of a pepsin-like enzyme in human and animal urines (uropepsin) has been reviewed by Bucher (44). Present evidence indicates that the substance actually present in blood and urine is pepsinogen. This is suggested by its alkaline stability which disappears after conversion to the active enzyme by exposure to an acid medium. The gastric origin of uropepsin has been confirmed in a recent study (45) in which it was found that total gastrectomy in cats causes the enzyme to disappear from the urine in two to six days following the operation. Gastrectomy in rats caused uropepsin to vanish from the urine within 24 hours (46). The enzyme may be of considerable clinical interest in the study of gastric function; peptic activity of the urine was found to be markedly diminished in patients with pernicious anemia as compared to normal subjects (47).

Northrop (48) has found that pepsin may be crystallized from 20 per cent ethanol although the enzyme is soluble in 65 per cent alcohol. Bourdillon (49) has described the isolation from commer-

cial pepsin of a nondialyzable crystalline polypeptide (peptophan) which is resistant to pepsin and which is stable in 0.1 *M* HCl and NaOH. This unusual protein contains 16.1 per cent nitrogen and does not give tests for tryptophane, carbohydrate, sulfhydryl groups, and phosphorus. This investigator (50) has also used pepsin to digest beef and horse muscle, and from both sources has isolated pepsin-resistant crystalline proteins which he has called peptomyosins.

A number of methods have been described for the assay of pepsin, mainly for use in clinical applications (51 to 54).

Buchs (55) has observed that in human gastric juice, and in preparations of commercial and crystalline pepsin, there is an additional proteinase which he has identified as a cathepsin. This enzyme has been differentiated from pepsin by its greater stability at high temperatures. Gastric juice which has been heated to 70°C. shows little or no peptic activity and yields a pH activity curve which shows an optimal action on edestin at pH 3.5 and on other proteins at slightly higher pH values. The characterization of this enzyme as a cathepsin is indicated by the strong activation which is produced by sulfide and by cyanide. Buchs has ascribed a major role in gastric digestion to this new enzyme. Further study and confirmation of these observations appears to be indicated particularly since crystalline pepsin has been shown to hydrolyze certain peptides and peptide derivatives at pH values near the optimum of this new enzyme (26).

Schales *et al.* (56) have found that pepsin is inhibited non-competitively by a variety of carbonyl reagents such as hydroxylamine, semicarbazide, hydrazine, dimedon, and bisulfite, but not by thioglycolic acid. The hydrazine inhibition was observed not only with several protein substrates, such as egg-white, bovine albumin and casein, but also with carbobenzoxy-L-glutamyl-L-tyrosine. In view of the low degree of inhibition produced by some of the reagents, it would be of considerable interest to ascertain the effect of these inhibitors on the catheptic activity described by Buchs.

Trypsin.—Schwert *et al.* (57) have found that crystalline trypsin hydrolyzes ester linkages of amino acid derivatives where the esters possess the same configuration as the specific amides which are split by this enzyme. Thus, α -benzoyl-L-arginine methyl ester and α -*p*-toluenesulfonyl-L-arginine methyl ester are hydrolyzed

about 60 times faster than the corresponding amides. Both the esterase and amidase activities appear to be mediated by the same active centers of the enzyme as indicated by studies of various methods of partial and complete enzyme inactivation.

Methods have been described for the estimation of tryptic or proteolytic activity of pancreatic secretion (54, 58, 59).

Trypsin inhibitors.—The crystalline trypsin inhibitor from soybean described earlier (60) has now been found by Kunitz (61) to form a stable crystalline compound with trypsin. The compound is a globulin with a minimum solubility at pH 5.2; it possesses practically no proteolytic or inhibitor activity. Heating at pH 3.0 denatures the compound, but on cooling the denaturation is reversed. The compound contains about equal weights of the inhibitor and trypsin. The reaction appears to consist of a neutralization of the free amino groups of the trypsin by the free carboxyl groups of the inhibitor.

The mechanism of trypsin inhibition has also been studied by Borchers *et al.* (62), who conclude that the soybean inhibitor has no effect on the substrate and that it acts in a noncompetitive manner. Their findings are in substantial agreement with the formation of the trypsin inhibitor complex found by Kunitz.

It is reported by Bowman (63) that there are several different trypsin inhibitors present in soybeans. Trypsin inhibitors have also been found (64) in the seeds of many other members of the Leguminosae, but not in all seeds of this family. They have not been detected in the nonlegumes studied.

The soybean inhibitor is not affected by pepsin, trypsin, papain or "crepsin;" it is inactivated by ficin (65). The same fractions of soybean meal that contain the growth inhibitor contain the trypsin inhibitor (65). It is, therefore, assumed that the two factors are identical, and that the trypsin inhibitor acts by preventing the complete digestion of a part of the soybean protein. However, in the case of the lima bean trypsin inhibitor, Klose *et al.* (66) have found that the growth of rats is prevented even when a completely hydrolyzed protein (casein) is fed. Thus, in this instance, the major growth inhibition must be due to some effect not directly related to tryptic hydrolysis of the dietary protein.

The trypsin inhibitor of egg white has been identified (67) as the water-soluble ovomucoid. The protein contains 21.6 per cent carbohydrate and 13.3 per cent nitrogen; it has an average molec-

ular weight of 29,000. The mechanism of trypsin inhibition by ovomucoid has been intensively investigated (68). In contrast to the soybean inhibitor, ovomucoid has little or no action on other proteolytic enzymes. Acetylation of most of the amino groups inhibited neither the inhibitor nor trypsin. However, the acetylated trypsin was no longer susceptible to inhibition by ovomucoid or its acetyl derivative. The proteolytic activity of trypsin was independent of its most reactive carboxyl and phenolic groups, but depended on the integrity of amide, guanidyl, hydroxyl, and indole groups. It appears that trypsin combines with the substrate through groups other than amino but combines through its amino groups with the acid groups of the inhibitor. Kinetic studies indicated that inhibition of trypsin by ovomucoid is of the noncompetitive type, in agreement with the evidence that the substrate and inhibitor combine with different active groups of trypsin.

A crystalline trypsin inhibitor with anticoagulant properties has been obtained from pancreas (69). It differs in crystal form and conditions of crystallization from the pancreatic inhibitor described earlier (70).

Chymotrypsin.—Jacobsen (71) has made a detailed study of the mode of activation of chymotrypsinogen by trypsin. At 0°C., tryptic hydrolysis of one peptide bond of chymotrypsinogen yields π -chymotrypsin which is 2 to 2.5 times more active than α -chymotrypsin. This π -chymotrypsin, which has not been isolated, is further hydrolyzed in two simultaneous competitive reactions, one leading by tryptic hydrolysis of one peptide bond to the formation of a δ -chymotrypsin with a specific activity 1.5 times that of α -chymotrypsin, and the other by an autolytic hydrolysis of three peptide bonds per π -chymotrypsin leading to the formation of the crystallizable α -chymotrypsin. At a high activation rate, the former reaction leading to δ -chymotrypsin is dominant.

Chymotrypsinogen has 23 carboxyl and 20 nitrogenous basic groups per molecule while α -chymotrypsin has 6 to 9 additional titratable groups. Since probably 4 peptide bonds are broken during the conversion, it is suggested that the greater part of the increase in titratable groups is due to the scission of peptide bonds in the interior of the peptide chain or chains of chymotrypsinogen. This is in agreement with the earlier conclusions of Kunitz (43). Lenti (72) has stated that the spontaneous cleavage of chymotrypsin liberates cystine.

The crystalline protein isolated from beef pancreas by Laskowski (73, 74, 75) has now been identified as a new form of chymotrypsinogen (76). After activation with crystalline trypsin, crystalline chymotrypsin B was prepared. This new form is not identical with α -chymotrypsin since it shows a somewhat lower activity towards casein and may be crystallized by a procedure which is ineffective for α -chymotrypsin. The enzyme has been identified as a chymotrypsin by its ability to clot milk, and definitively by its ability to hydrolyze known synthetic substrates for other chymotrypsins (77).

The ability of chymotrypsin to exist in a variety of active forms seems to be unique for this enzyme. It should be recalled that in addition to the new protein described above (chymotrypsin B), Kunitz (78) had previously isolated three distinct crystalline forms which he designated α , β , and γ . The highly active chymotrypsins observed by Jacobsen and identified as π and δ give a total of six different active forms of this enzyme.

Schwert *et al.* (57) have found that the ethyl esters of carbobenzoxyglycyl-L-tyrosine and of carbobenzoxyglycyl-DL-phenylalanine are readily hydrolyzed by chymotrypsin. It will be recalled that the corresponding amides have previously been described as substrates for chymotrypsin (79). Benzoyl-L-tyrosinamide and benzoyl-L-tyrosine ethyl ester are also split by this enzyme (80), the latter about 300 times more rapidly than the amide.

Cathepsins.—Gutmann & Fruton (81) report the partial purification of an enzyme from swine kidney which hydrolyzes synthetic peptide derivatives previously found to be substrates for crystalline chymotrypsin. These preparations contain an endopeptidase and an aminopeptidase which, in the presence of cysteine, hydrolyze peptide bonds involving the carbonyl group of tyrosine or phenylalanine. The chymotrypsin-like action is differentiated from the trypsin-like activity of cathepsin II (substrate: benzoyl-L-argininamide) by the greater heat stability of the former. It is noteworthy that the partially purified enzyme hydrolyzes glycyl-L-phenylalaninamide at a rate about 30 times greater than the corresponding action of crystalline chymotrypsin on the same substrate. The swine kidney preparation hydrolyzes gelatin and β -lactoglobulin and the present evidence indicates that cathepsin II is involved in this action. The crystalline trypsin inhibitor of

soybeans has no effect on the hydrolysis of benzoyl-L-argininamide by cathepsin II, although it inhibits the hydrolysis of the same synthetic substrate by crystalline trypsin.

A manometric method has been described for following the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by catheptic enzymes (82). The method depends on the ability of a bacterial decarboxylase to liberate carbon dioxide from L-tyrosine, but not from the peptide derivative. This method has been utilized in a study of the reaction kinetics of swine kidney cathepsin I (83). The hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine follows a biphasic curve, with zero order kinetics in the early part of the reaction. One of the split products, carbobenzoxy-L-glutamic acid, acts as a competitive inhibitor of the enzyme. Over a limited range of substrate concentrations, the experimental curves agree fairly well with the results to be expected on the basis of the formation of an enzyme-substrate complex.

Cathepsin preparations from normal calf spleen and thymus (84), and from a rat lymphosarcoma (85), are inhibited by reagents which react with sulfhydryl groups (*p*-chloromercuribenzoate, iodoacetamide, etc.) as tested by their hydrolytic action on hemoglobin.

Zamecnik & Stephenson (86) report that aqueous extracts of a primary hepatoma induced by the feeding of *p*-dimethylaminoazobenzene hydrolyze benzoyl-L-argininamide more rapidly than do extracts of normal rat liver. This has been confirmed (87), and it has been found that the optimal action for extracts of liver and the hepatoma is at pH 6.2. A seven-day fast causes a fall of rat liver cathepsin and other enzymes which exceeds the proportionate loss of liver protein (88).

A proteolytic enzyme which is active on gelatin has been extracted from thyroid (89) [see also (90)]. It is reported that the activity is inhibited by iodine and by cyanide.

Racker & Krimsky (91) have found a factor in mouse brain which inactivates phosphoglyceraldehyde dehydrogenase, thus causing an inhibitory effect on the glycolytic activity of this tissue. The effect is increased by addition of ferrous sulfate, and is simulated by the addition of proteolytic enzymes such as trypsin or a cathepsin from beef liver. The inactivating factor loses its activity on dialysis against 0.1 *M* cyanide; it can be reactivated by the ad-

dition of ferrous sulfate and cysteine. It is suggested that the heat-labile factor may be an intracellular protease which is activated by ferrous ion and cysteine.

PLASMA ENZYMES

Although there is now some evidence that plasma proteinase (plasmin) differs in many respects from trypsin (20), it is of great interest that the crystalline trypsin inhibitor of soybean has a powerful inhibitory effect on the plasma proteinase and on blood coagulation (92). This anticoagulant effect of the inhibitor has been ascribed to its effect on thromboplastin (93, 94). It has been reported that α -tocopheryl phosphate has an anticoagulant action *in vitro* and *in vivo*, and is also a potent inhibitor of plasma protease, trypsin, leucoprotease, and, to a slight degree, papain (95).

The conversion by chloroform of plasminogen to plasmin which has been studied by Christiansen (96) appears to be due to the destruction of a plasmin inhibitor. After inactivation of the inhibitor, plasminogen is autocatalytically converted to plasmin. Protamine accelerates fibrinolysis by combining with the plasmin inhibitor as shown in dogs in peptone shock (97).

Iodination greatly decreases the hypotensive effect of trypsin while causing a smaller decrease in its proteolytic activity (98). Bowman (99) has found that the iodinated trypsin hastens the coagulation of recalcified plasma.

The nomenclature of the plasma proteinases and related substances has been discussed by Ferguson (100). The relationship of blood coagulation and proteolytic enzymes has been reviewed by Fredericq (101) and by Arvy (102).

In the past few years there has been a revival of interest in the peptidases of human serum (3, 103, 104). Fruton (3, 20) has suggested that the presence of peptidases in sera might be due to the liberation of these enzymes into the circulation in the course of the disintegration of lymphoid tissue. It has now been reported (105) that injection, in mice, of adrenal cortical extracts or of pituitary adrenotrophic hormone causes an appreciable rise in the serum peptidase level. In view of the known hormonal control of lymphoid tissue (106), it is suggested that the peptidase increase is due to liberation from these cells. However, it has been observed that injection of adrenotrophic hormone in humans produces only

slight increases in the level of some, but not all, serum peptidases under conditions where marked turnover of circulating lymphocytes occurs (107).

It has also been found, in an extensive study of normal and abnormal human sera, that the greatest increases in the level of certain serum peptidases occur in certain cases of anemia, particularly of the hemolytic type (108). Moreover, experimental hemolytic anemia in dogs, produced by injection of phenylhydrazine, causes a marked increase in serum peptidases. It is apparent from these observations that a portion of the serum peptidases may originate from disintegrating erythrocytes. In fact, preliminary observations have demonstrated the presence of peptidases in erythrocytes. From the foregoing, it is apparent that peptidases may originate in a variety of different types of cells.

BACTERIAL ENZYMES

Bidwell & van Heyningen (109) describe the preparation from culture filtrates of *Clostridium welchii* of a collagenase (*K*-toxin) which appears to attack only collagen and gelatin. The partially-purified enzyme (*circ.* 200-fold) is optimally active at pH 6.0 to 7.5 and is free of other toxins of the organism. A proteinase has been obtained by Kocholaty & Krejci (110) as an electrophoretically homogeneous protein from cultures of *C. histolyticum*. This protein which contains little tyrosine or tryptophane is activated by ferrous ion and sulfhydryl groups. With the activators, the enzyme hydrolyzes gelatin and clupein with a greater liberation of carboxyl than amino groups; it is inferred from this that imino peptide bonds may be attacked. Evans (111) reported that extracellular collagenase is produced by 30 strains of *C. welchii*, and also by some other species of *Clostridium*.

It has been found that the proteinase of culture filtrates of streptococci is derived from an inactive precursor (112). The conversion occurs under reducing conditions and is autocatalytically effected by the active enzyme or by trypsin. The streptococcal proteinase is inhibited by a specific antiprotease from immune horse sera (113).

Ramon *et al.* (114) report the production of an extremely active gelatinolytic enzyme by *Bacillus subtilis* when the culture medium contains bran, yeast, and dried malt.

PLANT ENZYMES

The proteolytic enzymes of milled fractions of wheat is the subject of a review by Howe (115). A study (116) of the distribution of proteolytic activity in resting cereals (wheat, barley, rye), using edestin as the substrate, has demonstrated a proteinase in fairly large amounts in the aleuronic cells; this is not present in measurable amounts in the endosperm or in the germ. Peptidase activity, using DL-alanylglycine as the substrate, shows a similar distribution.

Tracey (117) has found protease activity in the green leaves of tobacco and other plants using gelatin as a substrate. The activation of the enzymes by reducing agents and the pH optima suggest a relationship to the papain enzymes.

Hoover & Kokes (118) have observed that the optimal pH for the initial rate of papain digestion of casein is at pH 7 but the hydrolysis is much more extensive at pH 5. The hydrolysis by papain of 3 synthetic substrates shows pH optima at about pH 5.0 to 5.5. Thus, the action on the peptide derivatives is comparable to the action on the casein split products.

Dekker & Fruton (119) have utilized the synthetic optical specificity of papain in the formation of acyl-L-amino acid anilides to effect the resolution of DL-methionine. However, it has been observed (120) that papain effects the synthesis of carbobenzoxy-D- and L-fluorophenylalanyl hydrazides at about the same rate. In connection with the enzymatic resolution of amino acids, it is reported that commercial pancreatin causes the asymmetric saponification of the isopropyl ester of DL-methionine (121).

The degree of inhibition produced by various quinones is about the same for papain, urease, and catalase (122), but there is no correlation with the antibacterial action of these quinones.

Northrop (123) finds that papain and ficin do not digest living tadpoles and *Arbacia* eggs. *Arbacia* eggs develop normally in solutions of ficin or trypsin, but the cells become separated in papain solutions.

A sterile 2.0 per cent solution of papain is a nutrient medium for various bacteria (124). In addition, papain serves as an anti-coagulant when added to blood and gives a higher percentage of positive cultures of *Streptococcus viridans*. It is also stated that papain neutralizes the inhibitory effect of sulfonamides.

The kidney bean (*Vicia faba*) shows (125) a maximal protease

content at about the eighth day of germination. The presence of optima at about pH 2.0, 4.2, and 7.85 is taken to indicate the presence of three different proteases. Methods of extraction of a proteinase from germinated seeds of *Phaseolus aureus* are given (126).

Proteolytic enzymes have been described (127) in solutions of takadiastase. Evidence is given for differentiating between (a) a protease which attacks casein or gelatin; (b) distinct peptidases which attack leucylglycylglycine and other tripeptides; and (c) a peptidase which hydrolyzes leucylglycine.

ACTION ON PROTEINS

It is reported that the action of crystalline chymotrypsin on insulin (128) occurs in two stages: a rapid action leading to the formation of about 50 per cent nonprotein nitrogen followed by a second slow stage in which the nonprotein nitrogen rises to 100 per cent. Diffusion experiments indicate that the first material has an average molecular weight of about 4,000 as compared to 800 for the final soluble fraction. The biological activity of the insulin disappears at the end of the first stage. It is of interest that crystalline trypsin has no appreciable effect on insulin but acts on the chymotrypsin digestion products. The structure of the peptides obtained after proteolytic action on insulin is being actively pursued by Edman (129), Woolley (130), and Butler *et al.* (131).

Insulin is rapidly digested by pepsin at pH 2 (132). On standing at various pH values, material (plastein) is formed which is insoluble in trichloroacetic acid and in buffers. The formation of this material does not require active pepsin, and there is no evidence for the synthesis of peptide bonds. Butler *et al.* regard the precipitate (molecular weight of 12,000) as being an aggregate of the soluble split-products (molecular weight of 2,000).

Northrop (133) reports that when solutions of pepsin and trypsin are allowed to autolyze under special conditions, there is no evidence of increase in either peptic or tryptic activity. It is obvious that the plastein formed is not identical with the protein originally hydrolyzed.

Plastein formation has also been studied by the action of crystalline pepsin at pH 4.1 and 37°C. on the peptic digest of egg albumin (134). It is reported that the number of free amino groups decreased 25 to 50 per cent and that a corresponding amount of

plastein precipitated. The molecular weight of the plastein in phenol was about 300. It is suggested that a peptide ring structure was formed.

It has been claimed (135) that mushroom tyrosinase oxidizes proteolytic enzymes at the tyrosyl residues without producing inactivation of the enzymes. This has been questioned by Edman (136, 137), who finds that the oxygen uptake of these preparations can be explained by the oxidation of the nonprotein tyrosine and tryptophane. Moreover, it is observed that some decomposition occurred, particularly since trypsin and pepsin lost all of their activity although chymotrypsin was not inactivated. Sizer (138) has reinvestigated this reaction using not only the three proteolytic enzymes but other purified proteins as well. His newer results are in agreement with the earlier ones in that analyses for tyrosine on the proteins which had been oxidized by the tyrosinase and subsequently purified showed appreciable tyrosine oxidation. However, the oxidation of the autolytic products of the proteases observed by Edman was also confirmed.

Proteolytic enzymes have been utilized to demonstrate the protein nature of staphylocoagulase (139), and of paramycin (140).

In agreement with earlier work, native horse serum albumin is found (141) to resist proteolysis but is easily hydrolyzed after heat denaturation. Similarly, in the presence of urea, the protein is more readily decomposed by papain, but the protein again becomes resistant to digestion if the urea is removed by dialysis. Solubilized heat-denatured albumin resembles the native protein in its resistance to tryptic hydrolysis. It is reported (142) that those proteins that are most easily attacked by trypsin show the greatest rise in optical activity on heating.

The optical rotation of various proteins during peptic hydrolysis remains strikingly constant (143), whereas the filtrates obtained after trichloroacetic precipitation show marked increases in rotation. It is concluded that the fragments split from proteins by pepsin are relatively large and have the same rotation as the original proteins. However, tryptic digestion effects a great variation in rotatory power because it liberates free amino acids.

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NONOXIDATIVE, NONPROTEOLYTIC ENZYMES¹

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It has been, more or less, a tacit assumption that enzymes constitute only a small part of the protein of the living cell. Virtanen has pointed out that from our present knowledge of the quantities of enzymes it seems justifiable to conclude that in microorganisms practically all protein is enzyme protein. Therefore, the formation of an adaptive enzyme should correspond to a decrease in some other enzyme or enzymes, presumably of a related type. Lowering of the protein content of the cell by starvation should decrease primarily the amounts of adaptable (dispensable) enzymes, whereas the indispensable (constitutive) enzymes should maintain their activity. Virtanen & De Ley (1) have determined saccharase in *E. coli* which is, in this case, an adaptive enzyme, and catalase, a typically constitutive enzyme. By nitrogen starvation the protein content of the cells could be materially decreased. The saccharase content fell rapidly but catalase activity was only slightly affected.

Investigations by Bücher & Kaspers (2) on the photochemical splitting of carbon monoxide hemoglobin may throw light on the action of the protein carrier on the prosthetic groups of enzymes. Not only radiation absorbed by the hemin splits off carbon monoxide, but also radiation absorbed by the protein. In the photo-inactivation of urease the effect is the same, if the quanta are absorbed by a few tyrosine or tryptophane residues or by all amino acids. It is suggested that transmission of absorbed activation energy may be one of the causes of the chemical activation of prosthetic groups by the carriers. Attention is called to a paper by Veldstra (3) on the interaction of "ergones" and substrates.

Rothen's experiments on "enzyme action at a distance" were mentioned in Vol. XVII of this *Review*. New experiments (4) have been published which are thought to exclude the possibility of diffusion of the enzyme through the protecting layers. Mettler, however, points out (5) that the antigen films are not of constant

¹ This review covers approximately the period from December, 1947 to December, 1948.

thickness but may contain protuberances as high as 200 Å, which would account for Rothen's results without assumption of long range forces.

Certain papers deal with the localization of enzymes in cells. Suomalainen (6) concludes that certain hydrolases are localized on the yeast cell surface, others within the cell. Rothstein & Meier (7) have demonstrated that, when living yeast acts on adenosinetriphosphate (ATP) containing radioactive phosphorus, all of the P^{32} is recovered in the medium, none in the cells, indicating that the enzyme is on or in the surface of the cell.

A new enzyme which splits halogen from certain organic halides is described by Heppel & Porterfield (8). Bernheim (9) has found an enzyme hydrolyzing hydantoin but having no action on hydantoin derivatives. Another new enzyme catalyzing the hydrolysis of cyanate into carbon dioxide and ammonia has been discovered by Holtham & Schütz (10). Meister & Greenstein (11) report that extracts of liver and kidney split 2,4-diketo acids; 2, 4-diketo valeric acid yields pyruvic and acetic acid.

Dawes (12) has given a review on the theories of tryptophanase action. The best explanation seems to be that of Wood, Gunsalus & Umbreit (13) stating that the action is nonoxidative, yielding indole, pyruvic acid, and ammonia.

Esterases.—Horse liver esterase has been crystallized by Safwat Mohamed (14). Engel & Bretschneider (15) have compared the amounts of esterase (protease, amylase) and number of mitochondria in wheat grains. No correlation was found. Wheat germ esterase has been studied by Singer & Hofstee (16). The reaction is of zero order in presence of excess of substrate. A correlation was found between the Michaelis-Menten constants and molecular size of the substrate. The inhibition by arsenicals, etc. (17) is also correlated with the size of the substrate molecules.

The affinity of pig pancreas lipase to lower glycerides in homogeneous solution and to triacproin in heterogeneous solution has been studied by Schønheyder & Volqvartz (18, 19). Boissonnas (20) uses Tween 20 as a convenient water soluble substrate for pancreatic esterase. The lauric acid liberated is titrated with phenol red. The author (21) has some doubts about the crystalline enzyme of Bamann & Laeverenz (22). New methods for extraction and precipitation are described. After seventeenfold purification eight proteins were present. More than one esterase is supposed to be

present in pig pancreas. Inactivation by fluorophosphates has been studied by Webb (23) and the action on glycerol esters with hippuric acid by Fodor (24). According to Lineweaver *et al.* (25) tributyrinase is found in hen's eggs, both in white and yolk. Enzymes splitting tributyrin and olive oil were found by Mansour-Bek (26) in the stomach juice of *Lamellibranchia*. Occurrence and specificity of ester-splitting enzymes in insects has been investigated by Fodor (27). The tributyrinase content of rat blood plasma on fat-free diet is strongly decreased (28); the cholinesterase content is not affected. Oleic acid ester of sorbitan stimulates growth of tubercle bacilli except in the case of very small inocula (29). The inhibition then observed is due to occurrence of free fatty acid. Serum counteracts this inhibition but contains, on the other hand, an esterase, the action of which must be eliminated to permit growth of the organism.

Acetylesterase.—Jansen *et al.* (30) have studied the esterase of citrus fruits. It is a true esterase, having no action on olive oil. It splits all acetic acid esters, but N-acetyl ethanolamine, N-acetyl glucosamine, and acetyl phosphate are not attacked. Acetylcholine is attacked but eserine does not inhibit the hydrolysis. The enzyme is specifically inhibited by fluorophosphate (31, 32), but not by fluoride. The inhibition does not depend on sulfhydryl groups. The substrate competes with the inhibitor, which suggests that the inhibitor combines with a substrate-binding group.

Bovet Nitti (33) characterizes the esterase in cobra venom as an acetylesterase since it splits not only choline esters but also other lower esters of acetic acid. It is inhibited by myristicyl choline, which has no action on serum cholinesterase.

Esterase activity of proteolytic enzymes.—Schwert *et al.* (34) have made the very interesting observation that proteolytic enzymes display a typical esterase activity toward certain esters of amino acid derivatives. This activity seems to be a general attribute of proteolytic enzymes. Crystalline trypsin, chymotrypsin, and carboxypeptidase have been investigated (35). Owing to the optical specificity pure antipodes of certain amino acids can be obtained by enzymatic degradation of suitable esters (36, 37).

Lecithinases.—Macfarlane & Knight have shown previously that the α -toxin of *Clostridium welchii* is a lecithinase. New experiments (38) show that the enzyme splits sphingomyelin and lecithin under formation of phosphorylcholine. Culture filtrates from *C.*

oedematicus contain a lecithinase of the same type (39). The lecithinases from *C. oedematicus* types A and B are immunologically different and, in turn, different from that of *C. welchii*; they are probably identical with the γ - and β -toxins of *C. oedematicus*. Toxins from certain strains of *C. welchii* modify the surface of red cells, thereby rendering them unable to agglutinate with members of the influenza virus series. The factor is an enzyme but not identical with the α -toxin (=lecithinase) (40). Filtrates from *C. sordellii* also contain a lecithinase.

A "lysophospholipase" (lecithinase B) from *Penicillium notatum* has been described by Fairbairn (41). It liberates saturated fatty acids from lysophospholipids, whereas the "phospholipase" (lecithinase A) splits off unsaturated fatty acids from lecithin and cephalin.

Cholinesterase.—According to Hawkins (42) folic acid has no role in the breakdown of acetylcholine. The cholinesterase from electric tissue has been purified by Rothenberg & Nachmansohn (43); no change in its specificity was observed and the enzyme seems to be enzymatically homogeneous. Zeller (44) has found cholinesterase in the dried venoms of 19 species of the *Colubridae* but not in venoms of more than 20 species of the *Viperidae*. A method for the histochemical demonstration of cholinesterase has been devised by Gomori (45).

Mackworth & Webb (46) have studied the inhibition of cholinesterase by diisopropyl fluorophosphate. The inhibition increases with time, is irreversible, and not dependent on substrate concentration. Pseudocholinesterase is inhibited in much the same manner. Nachmansohn *et al.* (47, 48) have found that the toxic effect of diisopropyl fluorophosphate is probably due to its action on brain cholinesterase. The same conclusion is reached by Freedman & Himwich (49). Jones, Meyer & Karel (50) have tested 42 organic phosphorus compounds and found their toxicity to be a function of their potency as cholinesterase inhibitors. Goldstein (51) points out that molar potency and combination ratio do not adequately describe an inhibitor. Classification of the inhibition as reversible or irreversible, and competitive or noncompetitive, is necessary. The action of β, β' -dichlorodiethyl-N-methylamine has been studied by Adams & Thompson (52) and compared to the action of eserine and diisopropyl fluorophosphate. Little (53) has found the mouse brain to contain two different "fractions" of

specific cholinesterase. Scheiner (54) gives directions for preparation of "true" cholinesterase from blood.

Augustinsson (55) has published a comprehensive study of the cholinesterase activity of various tissues and an extensive review of the older literature (692 references!). Esterase activity of serum and erythrocytes, brain, muscle, liver, kidney, and intestine of various vertebrates, of different tissues of certain invertebrates and of blood from *Helix* and *Spirographis* has been studied in respect to a series of choline esters, acetylsalicylic acid, tributyrin, and ethyl acetate as substrates. Occurrence, specificity, kinetics, and inhibition by various substances have been investigated. The results have been discussed by applying the Michaelis-Haldane interpretation. The cholinesterases are regarded as a "family" of related enzymes with widely differing properties. A tentative classification is based on the activity-substrate concentration relationships.

Phosphatases.—A micromethod for the determination of phosphatase in 0.01 ml. serum has been described (56). Some new substrates have been introduced (57). A method for quantitative determination of the enzyme in tissue suspensions is indicated by Aebi (58). Sarles (59, 60) has attempted the separation of acid and alkaline phosphatase from liver. Roche *et al.* (61) have tried to use the solubility curves in water-acetone mixtures to identify the active fractions of several phosphatase preparations. Roche & Sarles (62) report that the affinity of animal alkaline phosphatase to β -glycerophosphate is different for enzymes from different tissues whereas enzymes from the same organ of different animals have very nearly the same substrate affinity. Bone phosphatase has an exceptionally high affinity, which should be of importance for its supposed role in ossification.

Euler & Fonó (63) have studied the action of alkaline phosphatase of intestine on different substrates. They conclude that different enzymes split glycerophosphate, ribo- and desoxyribonucleic acid. β -Glycerophosphatase from intestinal mucosa of calf has been purified by Euler *et al.* (64); dialysis at pH 4.5 reduced the activity to one-fifth; addition of heat-inactivated enzyme almost doubled the activity.

Thoai, Roche & Roger (65) find that dialysis completely inactivates intestinal alkaline phosphatase. The enzyme is reactivated by magnesium, calcium, iron, manganese, and zinc ions after in-

cubation with alanine. The effects are observed only with highly purified solutions. The authors consider it possible that alanine "renaturates" denatured enzyme. Inactivation of intestinal phosphatase by storing has been investigated by Fischer & Greep (66). It is concluded that the enzyme is a metal protein and that the effects investigated involve the specific nature of the metal group. Euler & Fonó (67), working with the same enzyme, have separated enzyme and coenzyme (or rather an activator) by an adsorption technique. The concept of a dissociable prosthetic group for alkaline kidney phosphatase is rejected by Schales & Mann (68). Their experiments seem to show that activators and not a prosthetic group are removed by dialysis. Protein denaturation may be mainly responsible for the inactivation during dialysis.

The effect of activating and inhibiting substances on kidney phosphatase has been investigated by Aebi & Abelin (69). The authors point out that experiments of this kind should be made over the whole pH range of the enzyme in question. The effects of reagents specific for amino groups have been tested by Roche & Abul-Fadl (70). Montalenti & de Nicola (71) report experiments on the action of mustard gas (not mustard oil as incorrectly stated in the English summary) on phosphatases in cymoid oocytes. They have also studied the phosphatase distribution in certain gonads. The action of amino acids on various phosphatases has been investigated by Bodansky (72).

Zittle *et al.* (73) report that the hydrolysis of mono- and diesters and mono- and polynucleotides by calf intestinal mucosa is inhibited to different degrees by sodium arsenate. The action on mononucleotides is practically completely inhibited. Courtois & Bossard have shown previously that phosphatases are strongly inhibited by molybdate. Bossard (74) finds this inhibition to be rather specific for phosphatases. Fleming & Peczenik (75) have noted that toluene extracts of urine inhibit the alkaline phosphatase in tumors; stilbestrol has no effect.

The existence of a liver phosphatase specific for glucose-6-phosphate has been demonstrated by Broh-Kahn *et al.* (76). Galactose-1-phosphate is not attacked. Glucose-1-phosphate is probably not dephosphorylated but is rapidly converted to the 6-phosphate. The enzyme is probably not identical with the ordinary acid or alkaline phosphatases.

Experiments by Flock & Bollman (77) suggest that serum alkaline phosphatase in the rat is supplied from the small intestine by way of the lymph. Naganna & Menon (78) have found the erythrocyte pyrophosphatase to be practically inactive without magnesium. Inhibition by certain reagents suggests the existence of essential sulfhydryl groups in the enzyme.

Pyrophosphatase and monoesterase in the hepatopancreas of *Helix* have been investigated by Dessaux (79). In both cases three peaks were observed in the pH-activity curves, which causes the author to question the existence of specific phosphatases in the organ.

Experiments by Kornberg (80) on the cleavage of di- and triphosphopyridine nucleotide, flavin-adenine dinucleotide, and ATP suggest that only one enzyme is responsible. The pyrophosphate bond is the site of cleavage of diphosphopyridine nucleotide (DPN) by kidney preparations (81). The action is greater in respiring systems, which may indicate that the reduced form of DPN is more easily split. Inhibition of diphosphopyridine nucleotidase by α -tocopheryl phosphate is reported by Govier & Jetter (82). Investigations by Plumel (83) seem to show that three different phosphodiesterases exist in swine serum. Paget & Vittu (84) have found four different phosphomonoesterases in different *Salmonella*. Helferich & Stetter (85) have purified the acid phosphatase of potato: a 3800-fold increase in activity was reached. Fluoride ions inhibit and magnesium has no influence on the inhibition. Reagents which eliminate manganese ions have no influence on the activity. A yeast phosphatase with pH optimum at 8.5 to 9.1 is described by Hoffmann-Ostenhof (86); magnesium is not necessary for activation. Schmidt *et al.* (87) state that the phosphoric acid released from yeast nucleic acid by prostatic phosphatase during short incubation originates in the pyrimidine nucleotides.

Hydrolysis of ATP.—The interaction of ATP and myosin consists of an initial "response" (drop in viscosity) and a "recovery" due to enzymatic degradation of ATP [Mommaerts (88)]. Bailey & Perry (89) believe that ATP competes with actin for sulfhydryl groups in the myosin. The action of certain ions, especially magnesium, on adenosinetriphosphatase has been studied by Mommaerts (88) and by Braverman & Morgulis (90). Torda & Wolff (91) find that acetylcholine increases and caffeine, *d*-tubocurarine, and potassium ion decrease the activity in concentrations inducing

muscle contraction. Adenosinetriphosphatase in nerves is almost completely confined to the sheath, which has a very high activity [Libet (92)]. Nachmansohn *et al.* (93) have tested the hypothesis that adenosinetriphosphatase is related to the electrical activity of the nerve. It was found, however, that eserine and diisopropyl fluorophosphate do not affect the adenosinetriphosphatase when electrical activity is abolished. Banga *et al.* (94, 95) state that when pure myosin acts upon ATP, one phosphate group is split off. The solution then contains adenosinediphosphate (ADP) and a dinucleotide pentaphosphate (DNP). If ADP-isomerase (protein II) is added at this stage, phosphate and ammonia are split off with the formation of inosinic acid. If protein II is present from the beginning, inosinic acid is formed very quickly, but ADP and DNP cannot be intermediates because they are attacked very slowly. The action of myosin plus protein II is regarded as evidence for the Barrenscheen formula of ADP (termed ADP₂ as opposed to ADP₁, the Lohmann formula). Adenosinetriphosphate can also be deaminated without dephosphorylation.

Kielley & Meyerhof (96) have found an adenosinetriphosphatase in muscle, hitherto overlooked because of its instability. This very active enzyme can be completely separated from myosin. Hydrolysis of ATP with homogenates of certain tissues has been investigated by Herrmann & Hartman (97). Krishnan (98) has purified apyrase from potato. The enzyme splits off two phosphoric acid groups from ATP. Enzymes in the skin are said to dephosphorylate ATP completely (99); the enzyme content was significantly higher in tumors. The ophio-adenosinetriphosphatase of snake venom has been studied by Zeller (100). All species tested contain the enzyme which liberates only one mole of phosphoric acid from ATP.

Phosphotransferases.—Enzymes capable of phosphate transfer without participation of ATP are designated as phosphotransferases. Axelrod (101) reports that such enzymes have been found in citrus fruits, apples, human urine, and takadiastase, but not in the sweet potato, pear, or onion; kidney alkaline phosphatase is inactive as a transferase. Aryl phosphates are good donors, aliphatic alcohols are acceptors. The ratio of phosphate transferred to substrate cleaved increases with decreasing pH; it is independent of donor concentration but increases with acceptor concentration. The same effect was observed by Appleyard (102) for prostatic

phosphatase. By using labelled phosphorus, Axelrod (103) was able to show that the phosphorus transferred does not pass through the inorganic state.

Phytase.—Courtois *et al.* have studied phytase and its relation to other phosphatases. In wheat bran (104, 105) and certain seeds (106) a true phytase is mixed with an ordinary phosphatase which is inert toward phytin but is active toward lower inositol esters. No relation was observed between phytin content and phytase content (107). Animal alkaline phosphatase, urinary acid phosphatase, and takaphosphatase split inositol monophosphoric acid and, less easily, triphosphoric acid, but not the tetra-, penta-, and hexaphosphoric acids (108). Addition of phytase to a rat diet containing phytin did not improve the utilization of the ester (109). Phytase in certain seeds has been determined by Fontaine, Pons & Irving (110). The ability of dried yeast to produce rachitic symptoms in pigs was earlier ascribed by Hoff-Jørgensen to a specific phytase inhibitor. De Man (111) has found, however, that the inhibition is caused by inorganic phosphate, which is admitted also by Hoff-Jørgensen.

Phosphoglucomutase.—Schlamowitz & Greenberg (112) have obtained preparations of phosphoglucomutase free from phosphorylase and isomerase. A cyclic diester is proposed as an intermediate in the reversible conversion of glucose-1-phosphate to 6-phosphate (113). Caputto *et al.* (114) report that the conversion requires a thermostable coenzyme. Isolation of a crystalline, electrophoretically homogeneous preparation is described by Najjar (115); magnesium ion is an activator, fluoride ion an inhibitor. The enzyme is inactive without cysteine.

Phosphamidase.—Hanahan & Chaikoff (116) have found an enzyme in carrots and fresh cabbage leaves which splits the ester linkage between the phosphoric acid and the nitrogen base of phospholipids.

Hydrolysis of polymetaphosphate.—Ingelman (117) has isolated from *Aspergillus niger* a highly polymerized substance containing 25 per cent phosphorus and 15 per cent sodium, probably a polymetaphosphate. Its breakdown by enzymes from microorganisms (117a) has been studied by viscosimetry, ultracentrifugation, dialysis, and orthophosphate determinations.

Nucleases.—Experiments by Laskowski & Kazenko (118) have shown that the nuclease and protease activities of a crystalline

protein from pancreas, described earlier, are due to impurities. Kleczkowski (119) and McDonald (120) state that the proteolytic activity of crystalline ribonuclease varies enormously in different preparations. A method for the preparation of protease-free ribonuclease is described (121). A crystalline enzyme from beef pancreas with great activity toward desoxyribonucleic acid has been isolated by Kunitz (122).

Inhibition of ribonuclease by penicillin is reported by Massart *et al.* (123). The antibiotic activity of penicillin is not ascribed to this fact alone, but it may be recalled that Krampitz & Werkman found penicillin to interfere with the dissimilation of cellular ribonucleic acid. Paramecin, the "killer" substance from *Paramecium aurelia*, is inactivated by various enzymes including desoxyribonuclease (124).

Glycosidases.—Sumner & O'Kane (125) have found that saccharase is precipitated by concanavalin A. Since this substance precipitates glycogen it is supposed that the saccharase is a polysaccharide-protein complex. The inactivation of saccharase by tyrosinase is explained by Sizer (126) as an oxidation of essential tyrosine groups in the enzyme. Inactivation and reactivation of saccharase and raffinase have been studied by Wagreich, Abraham & Epstein (127).

Veibel *et al.* (128, 129) have continued their studies on the enzymatic hydrolysis of β -galactosides and β -glucosides. β -Galactosidase does not attack β -glucosides. The β -galactosidase of alfalfa seeds is different from the enzyme in almond emulsin.

Estienne, Castagne & Bertrand (130) have determined lactase in different fungi; only very small amounts were found.

β -Glucuronidase.—Levy (131) concludes from experiments with menthyl glucuronide that the enzyme from ox spleen is purely hydrolyzing; synthesis of the uronide seems to require a different mechanism. Phenol glucuronide as a substrate is introduced by Kerr, Graham & Levy (132). Kerr & Levy (133) find, in contradiction to earlier observations, that enzymes from different organs may have different pH optima. Liver and spleen contain two enzyme "fractions." The high enzyme content of the uterus of infant mice supports the view that its activity is a measure of cell division. Two different enzymes in spleen are also reported by Mills (134). Levy, Kerr & Campbell (135) state that the in-

crease of enzyme activity of the liver found by Fishman is not due to adaptation but to tissue injury.

Fishman *et al.* (136) regard the high glucuronidase content of cancer tissues as evidence of the involvement of the enzyme in malignant growth. There is, however, no correlation between blood glucuronidase and the incidence of cancer (137). The possible function of the enzyme in "metabolic conjugation" is discussed (138). The inhibition by blood plasma is shown to depend on a heat-stable, nondialyzable molecule, possibly an antienzyme (139).

Pectic enzymes.—Gäumann & Böhni (140) find that the pectinase of *Aspergillus niger* is a constitutive enzyme in contrast to the unspecific pectase which is formed only in presence of substrate. The presence in tomatoes of a polygalacturonidase with unusual heat-resistance was demonstrated by McColloch & Kertesz (141). The tomato pectinesterase has been characterized by Pithawala *et al.* (142).

Hyaluronidase.—New methods for determination of hyaluronidase are described by Dalgaard-Mikkelsen *et al.* (143) and by Dorfman & Ott (144). The kinetics have been investigated by Dorfman (145). More than one hyaluronidase seem to be present in microorganisms [Rogers (146)].

Austin (147) attacks the current view of the role of the enzyme in fertilization. Female rats were killed when the copulation plug was found. Eggs were recovered which were, in many instances, surrounded by cumuli of normal appearance. Nevertheless, most of the eggs showed sperms within the vitellus.

Human blood serum contains a hyaluronidase inhibitor which, according to Dorfman, Ott & Whitney (148), is not an enzyme as previously claimed. Glick & Moore (149) state that the inhibitor is not an antienzyme since its action is independent of the origin of the enzyme. The inhibition by serum, salts, hyaluronic acid derivatives, etc., has been studied by Hadidian & Pirie (150) and by Tobin *et al.* (151).

Guerra (152) claims that the "spreading effect" of hyaluronidase is inhibited by salicylate *in vivo*. No effect on the enzyme was found *in vitro* [Pike (153)]. Lowenthal & Gagnon (154) conclude that the inhibitor is a conversion product of salicylate. They find that gentisic acid has no action, but that the corresponding quinone is highly active. Meyer, Ragan & Weinschelbaum (155) likewise find

salicylic acid without effect *in vitro*, but an inhibitor is present in urine from patients treated with salicylate. The inhibition is said to be due to gentisic and gentisuric acid. Inhibition by hesperidine derivatives is discussed by Beiler & Martin (156). According to Swyer (157) sperm hyaluronidase has no effect on capillary permeability and the spreading action is not inhibited by salicylate. Histamine increased the spreading action of hyaluronidase and was inhibited by salicylate. Glick & Grais (158) disclaim the occurrence of hyaluronidase in skin. The observed action is ascribed to bacteria. The occurrence of the enzyme in different kinds of tumors was investigated by Dux & Lacour (159). They find no relation between enzyme content and type of tumor.

A mucinase, active against several glandular mucins but without action on hyaluronic acid, was studied by Burnet *et al.* (160).

Amylases.—Viscosimetric methods for α -amylase determination have been published by Hultin (161) and Landis & Redfern (162); colorimetric methods by Redfern (163), Bernfeld & Fuld (164), and Hoskam (165).

Balls, Walden & Thompson (166) have indicated a simplified method for preparation of crystalline β -amylase from sweet potato. The enzyme is a protein, containing more tyrosine than usual but very little cysteine or cystine. The nitroprusside test shows that a large portion of the sulfur exists as sulfhydryl groups. Danielsson & Sandegren (167) have purified the amylases of barley and malt. β -Amylase, as well as α -amylase, accumulates in the albumin fraction. They behave identically in the ultracentrifuge. The electrophoretic mobility is practically the same for both enzymes between pH 4.4 and 8.2 and the iso-electric point, pH 5.75, is identical for both. Sedimentation and diffusion give a molecular weight of 54,000 (168). Considerable purification of malt α -amylase was achieved by Schwimmer (169); the enzyme was crystallized by Schwimmer & Balls (170). It is a protein, easily soluble in 40 per cent alcohol. It contains 13.4 per cent nitrogen, 0.01 per cent phosphorus, and 0.035 per cent iron. Myrbäck & Frostell (171) have demonstrated that the acid branch of the activity-pH curve is determined by the low stability of the enzyme in acid solution.

Human salivary amylase has been crystallized by Meyer *et al.* (172, 173). The activity is increased sixteenfold in a yield of 30

per cent after recrystallization. Bernfeld *et al.* (174) state that the enzyme is a phosphorus-free protein. The enzyme is activated by chloride and certain other ions, just as saliva. This seems to dispose entirely of the theory, proposed by Barmenkow (175), that the action of the ions consists in liberation of the amylase from an adsorption compound with mucin provided that the crystalline enzyme is not a compound of this kind, which appears unlikely.

The amylase from pig pancreas was crystallized by Meyer *et al.* as mentioned in Vol. XVII of this *Review*. Further work on the enzyme is reported by Fischer & Bernfeld (176). A simplified method for the preparation is described. The enzyme is a protein with 15.8 per cent nitrogen. It probably does not contain phosphorus, sulfur, or reducing sugar. Apart from the action of chloride there is no inactivation by dialysis. Trypsin has no action on the enzyme, but in impure preparations (177) a proteolytic enzyme is present which causes irreversible inactivation. The recrystallized amylase is quite stable. The crystalline amylase from human pancreas [Meyer *et al.* (178)] is a protein with very low solubility in water at pH 7 to 8.5. The solubility is high at pH 11 where the enzyme is stable. The enzyme seems to be identical with that from human saliva, but differs strongly from the amylase of pig pancreas. Wang & Grossman (179) report that secretin does not stimulate the amylase secretion of dog pancreas, but pancreozymin has a certain effect. Hecht (180) finds a high amylase concentration in rat blood, with no difference in blood amylase between normal and diabetic animals.

An interesting review on the preparation and industrial use of mold amylases has been given by Underkofler *et al.* (181). Methods for purification are described by Gates & Kneen (182).

Kneen & Beckord (183) have tested the amylatic activity of a great number of bacterial isolates. Four different types were found: (a) the saccharifying *subtilis* type; (b) the α -amylase type (commercial preparations); (c) *polymyxa* amylase; and (d) *macerans* amylase. The *subtilis* amylase has been studied by Di Carlo & Redfern (184). Considerable purification has been attained. The existence of essential sulfhydryl and amino groups has been proved. Purification and crystallization of bacterial amylase is reported by Meyer *et al.* (185). The enzyme is a protein, not identical with the pancreatic amylase but having the same action on starch. The

amylase from *B. polymyxa* has been studied by Rose (186). It is found in the culture medium and saccharifies starch to about 80 per cent.

Concerning the mechanism of β -amylase action all authors seem to be, on the whole, of the same opinion. The enzyme is supposed to attack chain molecules with 1,4- α -glucosidic linkages from the nonreducing end group, setting free maltose molecules. One conclusion would be that amylose, if containing only unbranched molecules, must be saccharified to 100 per cent. This is claimed to be the case by several authors. Bernfeld & Gürtler (187) dissolve amylose in sodium hydroxide and mix the solution, drop by drop, with a strong enzyme-buffer solution. Retrogradation does not have time to occur and the hydrolysis reaches 100 per cent. Cleveland & Kerr (188) have studied the degradation of corn amylose with β -amylase and examined the products at various degrees of hydrolysis. In the first half of hydrolysis only maltose and unconverted amylose seem to be present. It is concluded that when an enzyme molecule attacks an amylose chain, it remains in contact with this individual chain until it is completely converted to maltose. Much the same idea has been expressed by Meyer *et al.* in the case of phosphorylase (see below). The proposed mode of action is certainly not easy to understand from a physicochemical point of view. When the β -amylase acts on a branched molecule (amylopectin or glycogen) all free end chains with nonreducing end groups are saccharified. The action is stopped by the uttermost branching points (or other "anomalies"). Using the method mentioned above (187) Meyer *et al.* (189) have determined the yield of maltose from corn amylopectin (62 per cent) and potato amylopectin (59 per cent). Thus, considerably more than 50 per cent of the glucose units must be situated in end chains. This is thought to support the views of Bernfeld & Meutémédian (190, 191) on the synthesis of branched molecules by joint action of phosphorylase and isophosphorylase. The present writer is not convinced, however, that amylopectin can result from an equilibrium between the reversible actions of the two enzymes.

Claus (192) has studied the action of β -amylase on thick starch pastes at elevated temperatures. Hodge *et al.* (193) have tested the action of β -amylase on amylopectins of different origin. The amylopectins differ in phosphorus content, nature of bound phosphorus and iodine absorption. In alkali lability, optical rotation

and behavior against β -amylase no differences are found. Maltose is the only low molecular product. In some starches (corn and sweet potato) the authors presume the existence of an intermediate fraction between amylose and amylopectin.

Regarding the action of α -amylases the present author proposed, several years ago, the following theory: (a) The enzymes attack only 1,4-linkages; the 1,6-linkages remain in the limit dextrins and are the chief cause of limit-dextrin formation; (b) the α -amylases are endo-enzymes, i.e., they act preferably on 1,4-linkages far from end groups; (c) their action on an individual linkage (or the enzyme-substrate affinity respectively) is dependent, if the distance is small, on the distance of the linkage from end groups or from anomalies such as branching points, etc. It follows that the affinity of the enzyme to a substrate molecule, and thus the velocity of the hydrolysis, is dependent on the chain-length of the substrate. In the case of malt α -amylase the affinity to long chain substrates is very high (194), whereas the affinity to chains shorter than about eight units is very low. In the case of other α -amylases this effect of chain length on affinity is less pronounced, which is the explanation of the differences in the hydrolysis curves of various α -amylases. In the case of the degradation of amylose with malt α -amylase the results have been questioned by Bernfeld & Studer-Pécha (195), who attribute the form of the curve to retrogradation of the substrate. This view is not in accordance with further experiments in the author's laboratory (196). It should be pointed out that the method used by the Swiss authors has the disadvantage that, at low degrees of hydrolysis, the curve cannot be determined with any accuracy. Haworth *et al.* tried, a few years ago, to explain the action of the α -amylases by the assumption that the enzymes rupture the "polymeric links" (1,6-linkages) and that the unit chains so formed unite again in different positions. Criticism of this theory [Myrbäck (197)] has caused the English authors (198) to revise their opinion; they admit that the phase of the enzyme action under discussion consists in hydrolysis of normal 1,4-linkages in interior chains of the amylopectin.

Bernfeld & Studer-Pécha (199) have determined the Michaelis-Menten affinity constants for certain α -amylases. The values decrease in the order: pancreatic amylase, bacterial amylase, malt amylase. It should be pointed out, however, that the values found represent the affinity of the enzyme to high molecular substrates;

the affinities to short chain saccharides may be quite different. Bernfeld & Fuld (200) have compared the increase of the reduction value and the activity as determined by iodine coloration. The ratio was the same for the enzymes investigated. The conclusion that the action of the enzymes is identical appears somewhat daring, since only the initial phase of the reaction has been studied.

Formation of maltotriose (4-maltosido-glucose) by α -amylases is to be expected. Occurrence of a fermentable trisaccharide, presumably maltotriose, has been reported by Myrbäck & Lundén (201) and by Blom and co-workers (202, 203). Caldwell *et al.* have published investigations on the action of pancreatic amylase (204) and amylase from *Aspergillus niger* (205), which seem to support the theory of α -amylase action presented above. Corman & Langlykke (206) report that mold-enzyme preparations contain, beside the ordinary amylases, a "glucogenic system," producing glucose not only from maltose but also from higher saccharides such as starch itself. The reviewer has pointed out earlier that glucose is a primary product also in the case of malt α -amylase.

It should be mentioned that Sutra (207) considers all present theories of starch constitution unfounded. Without presenting evidence he regards all limit dextrins as reversion products.

Macerans amylase.—Kneen & Beckord (208) have found, as previously reported by Myrbäck & Gjörling (209), that the Schardinger dextrins formed by action of the enzyme from *B. macerans* gradually disappear again. This might be connected with the interesting observation by French *et al.* (210) that the action of the macerans enzyme is reversible.

Cellulase.—Saunders, Siu & Genest (211) have studied an extracellular enzyme from *Myrothecium verrucaria*, which hydrolyzes cotton. The main product is glucose.

Phosphorylases.—A review of the present knowledge on the biological synthesis of polysaccharides has been given by Hehre (212). Swanson & Cori (213) have tested hydrolysis products of polysaccharides for their action as "germs" in the synthesis by potato phosphorylase. Their activity depends on the molecular size of the saccharide and on the number of end groups. An end group has no activating power if less than three to four units removed from a branching point. Muscle phosphorylase is activated only by large molecules with many end groups such as glycogen. Hydrolysis causes rapid decrease in activating power. Dextran,

dissolved in alkali and neutralized, activated both potato and muscle enzyme. Cohn & Cori (214) have found that no exchange occurs between inorganic phosphate and glucose-1-phosphate in presence of muscle phosphorylase *a* or potato phosphorylase in absence of polysaccharide. Neither is an exchange observed with the phosphorus of adenylic acid in a complete reaction mixture, nor between glucose labelled with C^{14} and glucose-1-phosphate.

Bernfeld & Meutémédian (190, 191) have studied an enzyme "isophosphorylase," previously called "branching factor" (Cori) or "Q-enzyme" (Haworth *et al.*), which synthesizes 1,6- α -glucosidic linkages from glucose-1-phosphate in presence of a suitable "germ." The enzyme splits off the end groups of the limit dextrin of β -amylase as glucose-1-phosphate, thereby creating new "normal" end groups which can be attacked by β -amylase. Only terminal 1,6-linkages are ruptured by the isophosphorylase and only if the next linkage is a normal 1,4-linkage. The enzyme, therefore, has no action on native starch or dextran. The phosphorylases of potato, yeast, and muscle are strongly inhibited by phlorhizin, but the isophosphorylase is not. The activity ratio, phosphorylase/isophosphorylase, in a mixture of both enzymes can, therefore, be varied by addition of increasing amounts of phlorhizin. The iodine color of the polysaccharide formed varies under these conditions from blue through violet to brown. After long incubation, however, the iodine color is always brown, indicating the formation of a highly branched polysaccharide.

A high phosphorylase content of guard cells is reported by Yin & Tung (215).

Hydrolysis of amides.—Virtanen & Hamberg (216) point out that ammonia formation in the peptic digestion of zein and casein is not due to pepsin but to acid hydrolysis. Ammonia is formed in large amounts from zein without rupture of peptide bonds. Deamination of various amides has been studied by Bray *et al.* (217). Under standard conditions, the highest rate was found for *p*-nitrobenzamide. Greenstein & Leuthardt (218) have investigated the activation and inhibition of the hydrolysis of amides by phosphate and other ions. Inhibition of brain and kidney glutaminase by glutamic acid is reported by Waelsch & Owades (219).

Deamination.—Chargaff & Kream (220) have used paper chromatography to prove the occurrence of cytosine deaminase in yeast and *E. coli*. The methods may, with suitable modifications,

have wide applicability. Lichstein *et al.* (221, 222) report further work on the role of biotin in amino acid deamination. Biotin deficiency could be obtained in several bacterial species, accompanied by a decreased ability to deaminate serine, threonine, and aspartic acid. Addition of biotin or adenylic acid restored the activity.

Arginase.—Roche & Mourgue (223) have tested the activity of liver arginase against several guanidine derivatives; only L-arginine, DL-arginic acid, octopine, and guanidoacetyl glycine were split. The presence of guanido and carboxyl groups in the substrate is necessary but not a sufficient prerequisite. The length of the chain between these groups evidently plays an important role. Hunter & Downs (224) report studies on arginase inactivation by protein denaturants. Dounce & Beyer (225) have isolated nuclei from different tissues by an improved method and determined the arginase activity. Changes in the arginase content of liver and mammary gland of rats during pregnancy, etc., have been studied by Folley & Greenbaum (226). The possibility of a role of arginase in the defense of the organism against tumors is discussed by Bach & Lasnitzki (227), who find that slowly growing tumors have a higher arginase content than rapidly growing tumors. According to Roberts (228), a very significant increase in arginase activity occurs in epidermal carcinogenesis.

Glycocyaninase-creatinase.—Roche, Girard & Lacombe (229) describe an enzyme in *Pseudomonas ovalis* which splits guanidoacetic acid into urea and glycine. It has no action on guanidine, glycocyanidine, creatinine, etc., and very little effect on creatine. Kopper & Beard (230, 231) have extracted an enzyme from *Pseudomonas* strains (isolated from human urine) which degrades creatine and creatinine into urea, ammonia, carbon dioxide and other products. Two enzymes are probably present: a creatinine hydrazase yielding creatine, and a creatine hydrolase (or possibly oxidase).

Urease.—Myrbäck (232) has shown that the activity-pH curves of urease for different urea concentrations may be calculated from the Michaelis-Menten theory, if it be assumed that a basic group in the enzyme molecule with $K_b = \text{about } 5 \times 10^{-8}$ is responsible for the combination with urea (in a polar form). Deasy (233) states that high urea concentrations inhibit the enzyme in alkaline but not in acid solution. A urea-ammonia complex is supposed to be an inhibitor of the enzyme. Effects of arsenic derivatives on

urease and certain other enzymes are reported by Gordon & Quastel (234). Peterson, Harmon & Niemann (235) have found an increase of activity if a solution of crystalline urease is diluted with water containing hydrogen sulfide. This might be due to a dissociation of urease with increase in the number of reactive sites, or the effect might be due to dissociation of an enzyme-inhibitor compound.

Penicillinase.—A gram negative, rod-shaped bacterium, producing penicillinase was isolated from cultures of *P. notatum* by Brodersen (236). Properties of the enzyme are described.

Thiaminase.—The enzyme system in fresh water mussels causing hydrolysis of thiamine was investigated by Reddy, Giri & Das (237) and was found to contain two enzymes with different pH optima. Manganese ion is said to be an activator. Jacobsohn & de Azevedo (238) have studied the occurrence of the enzyme in different animal organs. Iodoacetic acid has only a weak action on the enzyme. Krampitz & Woolley have found previously that the enzyme is inactivated by dialysis but reactivated on addition of the dialysate. Engelhardt & Tatarskaya (239) ascribe this phenomenon to removal of a coenzyme of very high stability. It is widely distributed in the animal kingdom in muscle, liver, etc.

Transmethylation.—Borsook & Dubnoff (240) have found that, in liver, there are at least two methyl transfer reactions. One is dependent on oxygen (methylation of guanidoacetic acid and nicotinamide). The other transmethylation is independent of oxygen, namely the formation of methionine from homocysteine, homocystine, or homocysteine thiolactone by choline or betaine. Dubnoff & Borsook (241) have partially purified the transmethylase from liver or kidney of different animals. Dimethylthetin is a donor, one methyl group being transferred to homocysteine. In liver homogenate dimethylthetin is much more effective than betaine or choline. The transmethylation by these three donors is effected by different enzymes which can be distinguished by differences in stability. Du Vigneaud *et al.* (242) state that dimethylthetin is also able to replace methionine as a source of labile methyl groups in the diet of rats. Dimethyl- β -propiothetin is also an excellent donor (243).

Aldolase.—Aldolase from rabbit skeletal muscle has been crystallized by Taylor, Green & Cori (244). The substance is electrophoretically homogeneous over a wide pH range. The

isoelectric point is 6.05. Velick & Ronzoni (245) have determined its amino acid composition. All nitrogen was accounted for by 18 amino acids. The minimum molecular weight is 140,000. Basic groups are in excess over acid ones. Nevertheless, the isoelectric point in phosphate buffer is found to be on the acid side, which is explained as due to binding of phosphate ions (246).

A new method for the colorimetric determination of aldolase has been described by Dounce & Beyer (247). Determinations were made in various cells and organs. Racker (248) has found an enzyme in bacterial extracts which converts pentose-5-phosphate to triose phosphate. Crystalline aldolase from rabbit muscle condenses triose phosphate and glycolaldehyde to a pentose phosphate which is not identical with ribose-5-phosphate. An aldolase from peas has been studied by Stumpf (249) and compared in respect to properties, kinetics, equilibrium constants, and inhibition with muscle aldolase.

Enolase.—Bücher (250) has calculated the molecular weight of crystalline enolase from light scattering. The value found, 66,000, agrees very well with those calculated from sedimentation and diffusion constants or from the mercury content of the crystalline mercury compound. In electrolyte-free solutions the enolase molecule dissociates into four parts apparently equal in size. Association occurs if small quantities of electrolytes are added.

Fumarase.—Estimation and purification of the enzyme has been studied by Scott (251). The protein described earlier by Laki is not identical with fumarase. No indications of a coenzyme are found. Scott & Powell (252) find that the equation $K_1/K_2 = K_{eq}$ is not valid for the reverse reaction: fumarate + water \rightleftharpoons malate, which intimates that the reaction takes place in more than one step.

Aspartase.—An enzyme from *Pseudomonas fluorescens* is used by Virtanen & Louhivuori (253) for the quantitative determination of aspartic acid.

Carbonic anhydrase.—A rapid electrometric method for the determination of carbonic anhydrase has been devised by Wilbur & Anderson (254). Krebs (255) points out that the enzyme can be used in many cases to show, for instance, that carbon dioxide and not one of the ions of carbonic acid is the product of a certain reaction. It is shown that carbon dioxide is the true product of urease and carboxylase action. Ashby & Butler (256) have studied

the distribution of the enzyme in the central nervous system of the developing fetus. The presence of carbonic anhydrase in saliva from man and ox is demonstrated by Sand (257). It seems to have considerable influence on the properties of saliva. Öbrink (258) discusses the role of carbonic anhydrase for the permeability properties of the gastric mucosa.

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CARBOHYDRATE CHEMISTRY¹

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INTRODUCTORY

The past five years have seen remarkable developments in the qualitative and quantitative handling of small amounts of carbohydrates and their methylated derivatives. The reviewer has therefore decided to devote the present article to summarising these advances where they concern investigations of the structures of nonnitrogenous polysaccharides. He hopes that a critical résumé of this kind may prove of interest and use to the biological chemist since most textbooks and reviews tend to stress final results rather than the means by which these results were obtained. The reviewer wishes further to state that he has been privileged, through the kindness of the authors concerned, to read, and report on, several important papers which, owing to the delays in publication in the United Kingdom, are unlikely to appear in print before this article itself. It is perhaps interesting to recall that, despite so many advances in technique, methylation, originally conceived in the St. Andrews laboratory of Purdie & Irvine, remains an essential tool for all fundamental structural investigations.

THE PROBLEM OF POLYSACCHARIDE CHARACTERISATION

Investigators of complex carbohydrates are always faced by the question whether their material is a single chemical individual or a mixture. In practically every instance in this field the ordinary criteria of homogeneity applicable to small molecules cannot be applied to polysaccharides. A series of compromises have to be accepted. These are usually related to superficial properties such as elementary analysis, optical activity, behaviour towards precipitating agents, and empirical composition with respect to the component monosaccharide radicals. In the last respect improvements in the technique of uronic acid estimations have been reported by Dische (18) and by Tracey (57). Pentose analysis is also facilitated by the work of Militzer (47).

Another complication lies in that many polysaccharides,

¹ This review covers the period to October, 1948.

particularly those of the "branched" types, exist not as molecules all having the same molecular weight and composed of identical numbers of component radicals, but as collections of polymeric homologues, each based on the same chemical ground plan but displaying a range of molecular weights. For example, a number of highly purified glycogens examined by Bell *et al.* (11), from sedimentation-diffusion data, have mean molecular weights in the range 4.4×10^6 (mammalian liver), 2.8×10^6 (mammalian muscle), and 0.7×10^6 (*Ascaris lumbricoides*). Every sample was noticeably polydisperse and the molecules therefore appear to be composed of varying numbers of "unit-chains" each having an average length of about 12 glucosyl radicals. This problem, and related ones, have recently been reviewed elsewhere (9).

Apart from the plant gums, polyuronides and certain polysaccharides of microbiological origin, complex carbohydrates do not possess groups which ionise readily, and therefore cannot be subjected to the ordinary electrophoretic procedures, so valuable in the protein field. Isherwood (personal communication) has recently shown that it is possible to subject neutral polysaccharides to electrophoretic analysis by employing solutions in normal sodium or potassium hydroxides when the substances will migrate in the electric field. Isherwood finds that inulin (dahlia) several times recrystallised from water (45) migrates as an electrophoretically homogeneous substance, as does sucrose. On the other hand certain gums have been shown to contain more than one component and whole wheat starch likewise undergoes separation into two fractions.

To free its constituent monosaccharide radicals, the polysaccharide must first be hydrolysed by a method which should at the same time effect complete hydrolysis and destroy as little as possible of the sugars liberated. Generally speaking, it appears that sufficient attention has not always been paid to this problem from the purely quantitative aspect. Some scattered references relating to hydrolyses of glycosides and oligosaccharides have been collected by Pigman & Goepf (54). With regard to polysaccharides, insoluble substances, such as cellulose, can be very satisfactorily hydrolysed by cold treatment with saturated (0°C.) hydrochloric acid or 72 per cent sulphuric acid. Such treatments are, of course, destructive to polyuronides, pentosans, and fructosans. For water-soluble polyaldohexopyranosides, normal hydrochloric acid at

100°C. appears suitable, although mannans may require more drastic treatment. Polyaldopentopyranosides undergo some decomposition under the above conditions; here hot 3 per cent nitric acid is favoured. Polysaccharides such as arabans and fructosans based on furanose radicals require only relatively weak acids for complete and rapid hydrolysis; indeed carbonic acid is said to hydrolyse inulin [Drew & Haworth (19)]. When uronic acid and furanose radicals occur simultaneously in the same material, preferential hydrolysis of the furanoside links may result by simple boiling of an aqueous solution of the polysaccharide itself (35).

The construction of a "balance-sheet" of the sugars found after hydrolysis has long been a matter of great difficulty. This can now be carried out on the microscale by the various paper-chromatograms with the quantitative developments devised by Partridge (52), Hawthorne (33), Flood, Hirst & Jones (21), and Isherwood & Jermyn (41). The latter workers have speeded up the chromatographic separation by employing organic solvents of relatively low viscosity in the moving phase. A useful note on the identification of "spots" on the chromatogram by means of various sprayed reagents is due to Forsyth (22).

Hough, Jones & Wadman (39) have recently devised a conventional type of partition chromatogram using cellulose columns, which may be used on scales impossible with paper, but which yields comparable results. Previously Wolfrom and his school had described adsorption chromatograms using various minerals whereby a number of useful separations can be carried out (27, 43). Wolfrom (46) has also devised practical chromatographic separations of carbohydrates and related substances in the form of their acetates. An ingenious chromatographic device, which has not yet been applied to carbohydrates, is that of staining the column with a noneluting, U. V.-fluorescing dye. The presence of bands on the columns is indicated by changes in the degree of fluorescence. This work is due to Brockmann & Volpers (15).

Much work has been reported on the adsorption chromatography of azoyl derivatives of sugars, a procedure initiated by Reich (55). Technical difficulties make it probable that this type of separation will be largely superseded by direct cellulose or mineral chromatography of the unsubstituted sugars.

Chromatography does not distinguish between D- and L-isomers. The easy identification of individual sugars, however, has been

facilitated in a number of instances. Arabinose may be selectively precipitated, and estimated, as the benzoylhydrazone (36), while xylose is likewise separable and can be determined as its dibenzylidene dimethyl acetal (14). Fructose, in concentrated solution, may be detected as the methylphenylosazone; recent improvements in this technique are recorded by Neuberg & Mandl (49). The reviewer considers that this osazone is best characterised in the form of its acetate [cf. Percival & Percival (53)]. Bell has reported a number of qualitative separations of monosaccharides through their crystalline *iso*-propylidene derivatives, based on differential hydrolysis rates shown by substances respectively containing pyranose or furanose rings (7).

METHYLATION OF POLYSACCHARIDES

This is, of course, usually effected by dimethyl sulphate and alkali hydroxides upon aqueous solutions or suspensions. Sometimes Purdie's reagents (methyl iodide and silver oxide) have been used to supplement the methyl sulphate method but, in the case of at least one fructosan, this can lead to erroneous results as shown by Haworth, Hirst & Isherwood (31). In the polyglucose series it seems particularly difficult to effect complete methylation. Instances are recorded where the material analyses as though it were completely methylated, but when hydrolysed and the cleavage products are examined, it becomes clear that either the "found" methoxyl content of about 45.5 per cent is due to some error in analysis, or else loss of ether-methyl has accompanied hydrolysis. These questions have been considered by Freudenberg & Boppel (23), Freudenberg, Ploetz & Jakob (25), Hess, Schulze & Krajnc (38), and by Bell (8).

In attempts to complete methylation of resistant hydroxyl in simple carbohydrates, use has been made of metallic sodium in various media. Freudenberg & Hixon (24) first used sodium in ether or benzene (followed by reaction with methyl iodide), and Pacsu & Trister (51) successfully used this procedure both in ether and toluene. It is not known whether these reagents, under similar conditions, have been applied to polysaccharides. Muskat (48) introduced the use of sodium in liquid ammonia (with methyl iodide) as a means of etherifying glycosides, etc. Applications to polysaccharides have been made by Freudenberg & Boppel (23) and by Hess, Schulze & Krajnc (38), to choose from a number of

examples. The latter workers, as well as Schorignia (56), have criticised the use of sodium in liquid ammonia as leading to decomposition of the polysaccharide or loss of methyl groups.

Fear & Menzies first introduced the use of thallos hydroxide or ethylate and methyl iodide for the methylation of hydroxyl groups in simple substances (20). These reagents have been successfully applied by Hirst and his collaborators (31) to the polysaccharide field in instances where groups unstable to caustic alkali are present (e.g., uronic acid radicals) or where resistance to aqueous methylation is encountered.

The reviewer feels it necessary to emphasise that success in methylating a polysaccharide seems to depend on two factors. The first is the use of a large amount of reagents, a fact not always realised in past years. The second factor is the nature of the polysaccharide itself, e.g., inulin and grass levan can be completely methylated in one or two treatments with methyl sulphate and aqueous soda, while glycogen, after a score of such methylations still contains unsubstituted hydroxyl.

A frequent preliminary to methyl sulphate methylation is acetylation of the polysaccharides. The object of this treatment is to render the material soluble or dispersible in an organic solvent such as acetone or dioxane and thus facilitate "simultaneous deacetylation and methylation." It has sometimes been suggested that such preliminary acetylations lead to degradation of the polysaccharide molecules, but until exhaustive experiments are made on molecular weight determinations using methods which are not subject to erroneous interpretation it does not seem logical to make an ultimate decision. The use of ketene as an acetylating agent for carbohydrates has been only rarely applied (40, 58, 59). Since sulphuric acid appears necessary as a catalyst (except in the case of alginic acid) it may be that ketene acetylations are accompanied by some molecular degradation of the polysaccharide.

CLEAVAGE OF METHYLATED POLYSACCHARIDES

Two general methods are available. The first consists in treatment (usually with heat) with an alcohol (usually methanol) containing an acid catalyst (usually dry hydrogen chloride). The interradsical glycosidic links are alcoholised and mixtures of the α - and β -alkyl pyranosides and furanosides result, according to the conditions of the experiment.

The second procedure consists in aqueous acid hydrolysis of the interradsical links whereby free sugars are liberated. Here hydrochloric acid is the usual source of H^+ and it may or may not be necessary to add a nonaqueous solvent to promote initial solubility of the methylated polysaccharide. Bell (4) has found glacial acetic acid of service in the case of methylated glycogens, etc., and dioxane (with sulphuric acid) in the case of methylated fructosans (unpublished work). For polysaccharides based on the furanose radical, oxalic acid has frequently been used as hydrolytic catalyst.

It seems likely that treatment with too high a concentration of mineral acid may sometimes lead to partial demethylation of methoxyl groups. This has been noted by the reviewer in unpublished work during an investigation of the estimation of glycosidic methoxyl radicals after Freudenberg & Soff (26). [See also (8, 23).]

SEPARATION OF THE METHYLATED CLEAVAGE PRODUCTS

Early work indicated that volatility under reduced pressure and partition from water into organic solvents was related to the degree of etherification of a monosaccharide. It was not, however, until Macdonald (44), at St. Andrews, determined the exact partition of methylated glucoses between chloroform and water, and Haworth & Machemer (32), at Birmingham, determined the procedure for crude fractional distillation of tetra- and trimethyl methylglucosides that fundamental principles were laid for accurate analyses of methylated polysaccharides. Until recently exact data were available for the glucose series alone.

Analysis of mixtures of α - and β -methyl glucosides of both 2,3,4,6-tetra- and 2,3,6-trimethyl glucoses was simplified by Hirst & Young (37). Previously, two facts had been disregarded: (a) the refractive index of a methylated carbohydrate varies markedly with temperature and (b) the refractive indices of the α - and β -series of a glycoside are significantly different. These authors showed that n_D^{20} and $[\alpha]_D$ could be graphically related. Temperature control of n_D measurements in the sugar series therefore became obligatory. Bell (5) reported similar measurements for 2,3,4,6-tetramethyl- α - and β -methyl galactosides, work which led to the identification of L-galactose as a constituent of snail galactogen [Bell & Baldwin (10)].

Bacon, in a paper by Bacon, Baldwin & Bell (1), devised a

simple graphical method for analysing four component mixtures of 2,3,4,6-tetramethyl and 2,3,6-trimethyl glucoses in the form of their α - and β -methyl glucosides. There is no doubt that the observations of Hirst & Young and of Bacon could be successfully applied to other sugars, provided pure samples of the appropriate α - and β -methyl glycosides could be obtained.

Procedures concerned with fractional distillation necessitate the use of relatively large amounts of material, depending on the proportions of the minor components. This fact has provided a stimulus for investigation of small-scale methods. Certain workers have demonstrated separations of different methylated monosaccharides by adsorption chromatography of the azoyl derivatives but here technical objections exist. These are, chiefly, the difficulty of obtaining quantitative esterification, and in the case of reducing sugars, the formation of two or even four isomeric products. This last difficulty has been ingeniously circumvented by Boissonnas (13) in the instance of aldohexoses by preliminary reduction to the corresponding hexitols. By this means separation, e.g., 2,3,6- and 2,3,4-trimethyl glucoses, may be attained. The reviewer considers, however, that such methods will be superseded by paper or other methods of partition chromatography. For it has been shown by Hirst, Hough & Jones (34) in an exhaustive investigation that both qualitative and quantitative analyses of mixtures of methylated aldohexoses, aldopentoses, free sugars, and methylated and free ketoses as well can be carried out accurately by this method. For example 2,3,6- and 2,4,6-trimethyl glucoses can be distinguished as can 2,4- and 2,3-dimethyl xyloses. Sixty-two different sugars are examined in this paper. The methods of quantitative analysis used are suitable only for aldoses; estimation of methylated fructoses has been satisfactorily established by Miss Anne Palmer in the reviewer's laboratory, using modifications of Roe's method devised by Cole, Hanes & Loughman (personal communication).

As in the instances of the unsubstituted sugars, it is obligatory to identify the partially or wholly methylated sugars which have been identified on the paper chromatogram. Brown & Jones (16) have devised two techniques for separating certain methyl glycosides of methylated sugars, the first by adsorption chromatography in the conventional manner, and the second by use of trains of continuous extractors using partition between water and appropriate solvents.

In the glucose series Bell (6) has used partition chromatography on silica-water columns to separate small quantities of tetra-, tri-, and dimethyl sugars and considers that considerable practical advantage accrues from using the free sugars in place of their glucosides. Bell & Palmer (12) have devised a similar method for analysing methylated fructoses. The partition coefficients in this series necessitate the use of a different set of organic solvents. Difficulties can be experienced in the fructose series owing to the volatility of tetramethyl fructofuranose. This difficulty can, however, be overcome. One advantage of using the silica column is its flexibility; several grams of material can be handled without obtaining "mixed fractions."

END GROUP ASSAY BASED ON PERIODATE OXIDATION

The ordinarily accepted principles of periodate oxidations have been fully reviewed up to 1943 (42, 50). New application of these reactions to chain length determination of polysaccharides have opened up a new field of microanalytical technique of great value in biology. Barry (2) used periodate in an attempt to determine the chain length of laminarin which is considered to be based on the 1:3-glucosyl radical. In such a case only the free "end groups" possess adjacent hydroxyl groups and are subject to periodate attack, being expected to yield one mole of formic acid per terminal radical with the simultaneous reduction of two periodate. Barry determined the consumption of periodate and deduced a chain length. Barry & Dillon (3) also examined the insoluble resistant cell wall of yeast ("glucan") which is known to resemble laminarin in being based essentially on the 1:3-linked glucosyl radical. Their results pointed to a mean chain length of 28 radicals for glucan; on the other hand, using the methylation technique, Hassid, Joslyn & McCready found no evidence of appreciable amount of end groups in the form of tetramethyl glucose (30).

This oxidation work is open to criticism. Halsall, Hirst & Jones (29) have shown that "over oxidation" of the substrate can take place under certain circumstances, in which case Barry's chain length estimate may prove too small. Hirst and his collaborators, using a carefully controlled oxidative procedure, obtained end group assays on glycogen (28), amylose, and amylopectin (17) agreeing extremely well with the results of methylation experiments. In conclusion it must be noted that periodate oxidations

can be applied to chain length determinations only when an appropriate structure obtains in the polysaccharide. For fundamental structural investigation, methylation is in no way superseded.

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CHEMISTRY OF THE LIPIDS¹

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This review is limited to those aspects of lipid chemistry which are not more specifically treated elsewhere. Thus, the chemistry of the fat-soluble vitamins is dealt with separately in this volume as also is the metabolism of the lipids, while the chemistry of the triglycerides (fats) is exhaustively reviewed annually by a Committee of the American Oil Chemists' Society (1) and in the Annual Reports of the Society of Chemical Industry (2). Accordingly, certain branches of lipid chemistry have been selected and the elimination of others will permit of a more detailed consideration of the chosen fields. The period covered is the second half of 1947 and the first nine months of 1948, but for the sake of adequate discussion reference is sometimes made to earlier work.

Analytical methods.—The use of adsorption techniques in the analysis of all types of mixtures of organic compounds is proving so valuable that it is not surprising to find these methods being increasingly used in the study of lipids. The methods may find application either in the separation and isolation of the various constituents of a mixture, or may be used to analyse a mixture without the isolation of individual fractions. For certain classes of lipids, simple chromatography, as opposed to partition chromatography, has so far only permitted the latter type of operation, although progress is being made towards separation and isolation.

One such class is the fatty acids or their esters. Claesson (3) showed that mixtures of acids or esters can be separated on silica into groups containing unsaturated straight chain compounds, saturated straight chain compounds, and branched chain compounds respectively (in order of decreasing strength of adsorption from heptane), by means of displacement analysis using a solution of oleic acid as developer. However, the analysis of each of these groups required the use of a different adsorbent, e.g., activated carbon; and displacement analysis, with recovery of pure fractions, proved impossible (4), though frontal analysis enabled the com-

¹ This review has been prepared as part of the work of the Food Investigation Organisation of the United Kingdom Department of Scientific and Industrial Research.

position of the mixture to be calculated. This difficulty in eluting all the fatty acids, at least selectively, has been reported by a number of earlier workers, and even in recent publications adsorption techniques of this type with fatty acids and their esters have usually involved the loss of quite large proportions of lipid left adsorbed on the column. Thus Reinbold & Dutton (5, 6) obtained partial separation on alumina of the mixed glycerides of soybean oil, of the ethyl esters of the fatty acids of soybean oil, and of binary mixtures derived from ethyl stearate, oleate, linoleate, and linolenate. The maximum recoveries of total lipid achieved by any combination of solvents were 82.4, 90, and 87.5 per cent respectively. Mazumdar & Goswami (7) also achieved some separation of stearic and oleic acids, using alumina, magnesia, or carbon, but silica gel proved disappointing. Recently, however, Holman & Hagdahl (8) have reinvestigated the displacement analysis of the higher normal saturated fatty acids. Claesson's observation (4) that picric acid and myristic acid were adsorbed on charcoal independently of each other, the former very strongly, was turned to good account by demonstrating that picric acid could serve as a developer for mixtures of fatty acids and allow fairly quantitative displacement. However, apart from the first, each fraction was contaminated with the tail of the preceding one, and the authors drew attention to the need for much further study before the method could be applied generally to the analysis of mixtures of unknown composition.

Partition chromatography, on the other hand, has proved much more successful for this class of lipids and, in the reviewer's opinion, it also avoids the danger emphasized by the work of some authors, e.g., Trappe (9, 10), of chemical changes taking place on the column. Reported changes have included oxidation of fatty acids, splitting of triglycerides, and, with acidic adsorbents or solvents, transformation of sterols into hydrocarbons. Early application of this method to fatty acids was limited to those having appreciable solubility in water as well as in organic solvents, since water was used as the stationary phase. This excluded all fatty acids with more than five carbon atoms. Peterson & Johnson (11) extended the scope of the method to acids of up to 10 carbon atoms by employing fairly strong aqueous sulphuric acid, e.g., 28 to 36 *N*, as the stationary phase, adsorbed on Celite 545. Fatty acids of intermediate

chain length are far more soluble in sulphuric acid than in water. Various developers were studied. With certain combinations it was also possible to obtain good separation of capric and lauric acids, but only partial separation of higher acids, up to stearic. Butter fatty acids analysed by this method gave results agreeing well with those obtained by distillation. Ramsay & Patterson (12) have also extended the method to a similar extent by using methyl alcohol adsorbed on silicic acid as the stationary phase and an aliphatic hydrocarbon, e.g., *iso*-octane, as the mobile phase. These authors also studied the limiting amounts of one acid which could be detected in admixture with an adjacent homologue and found it to be as little as 0.5 to 1 per cent. Branched chain acids behaved like unbranched acids of the same number of carbon atoms. Subsequently Ramsay (13) showed that refractionation could separate *n*-butyric and *iso*-butyric acids. Whereas Peterson & Johnson collected aliquots of the eluate, Ramsay & Patterson detected the position of the various bands during elution by adding bromocresol green to the methyl alcohol. Scarisbrick *et al.* (14) have published a preliminary note of yet a third modification of the original method which extends its scope to acids of eight carbon atoms. They employed heavily buffered columns of silica and a mixture of chloroform and butanol as developer and, like Peterson & Johnson, collected samples of eluate for titrimetric examination. They also found the separation to be independent of any branching of the carbon chain. Boldingh (15) has extended the method to the esters of the higher fatty acids, C₁₂ to C₂₀, and adapted it to the filter paper strip technique. The stationary phase consisted of vulcanized rubber latex, and a content of 30 per cent of rubber still left the paper sufficiently porous for diffusion. The developer was methyl alcohol alone or mixed with acetone or benzene. The position of the ester spots was determined by parallel diffusion on another strip which was then stained by immersion in a solution of Sudan IV in 50 per cent aqueous acetone. Corresponding portions of the other strip could then be cut out for elution, and the fatty acids identified from previously-determined R_F values.

The difficulty of observing the separation as it is actually proceeding has probably tended to retard the application of chromatographic methods to lipids. A simple technique described by Claesson (16) is, therefore, of particular interest to workers in this

field. The zones on a column can be detected by observing differences in the refractive index of the solution in contact with the adsorbent, by noting changes in the angle of total reflection of light from the surface. A chromatographic tube of rectangular cross-section is employed. The method can be applied to filter paper chromatography by placing the paper under a sheet of glass.

Simple chromatography, although inadequate for fatty acids and their esters, has proved satisfactory for such lipids as hydrocarbons, sterols, and sterol esters. Hess (17) has described the separation and estimation of the free and esterified cholesterol in plasma or serum using an alumina column and selective elution. The total lipids were adsorbed from petrol ether solution, the ester fraction eluted with petrol ether containing 10 per cent of ethyl ether and then the free cholesterol with petrol ether containing 10 per cent of ethyl alcohol.

Simple chromatography of this type applied to the separation of phosphatides and other complex lipids has failed because of the impossibility of eluting some of the fractions. Thus, although Taurog *et al.* (18) were able by the use of magnesia to separate and recover the choline-containing lipids from a liver extract, the non-choline-containing fraction was left on the adsorbent. The reviewer's own experience has been similar, and it is his opinion that partition chromatography offers a much more promising technique for the separation of the various classes of phosphatides. Chargaff *et al.* (19) have applied it in the first place to the analysis of some of the products of hydrolysis of phosphatides and cerebroside, namely, the nitrogenous constituents and the reducing sugars. They employed the filter paper method. For the nitrogenous constituents they used two sets of strips, one for detection of choline by conversion of its phosphomolybdate to molybdenum blue, and the other for identification of primary amines either with ninhydrin or by the appearance of fluorescent spots on heating. Reducing sugars were detected by treating the paper with *m*-phenylenediamine. Observed under ultraviolet light, fluorescent spots marked the positions of the reducing sugars. Various developing solvents were tested. This technique enabled the authors to detect the presence of a hitherto unidentified constituent of the nitrogenous base fraction, which gave a reaction with ninhydrin and had an R_F value which placed it above serine in the series ethanolamine,

choline, serine, the R_F values of which decreased in that order. The method also showed that galactose was the only sugar to be found in the cerebroside of beef brain and spinal chord, and in several other lipid fractions.

Closely related to partition chromatography is partition in a series of tubes, the so-called "counter-current distribution" as developed by Craig (20). This method also would appear to the reviewer to offer considerable scope in the separation of lipid mixtures, especially in cases where adsorption of one or more constituents onto the supporting material of a partition chromatogram occurs. It is interesting to note that Craig's technique has been applied successfully by Sato *et al.* (21) to the separation and estimation of normal fatty acids from C_2 to C_6 .

There is a continuing interest in methods for the estimation of sterols. Delsal (22, 23) has described a micromethod for the colorimetric determination of free cholesterol, cholesterol ester, and lipid phosphorus, as well as of total proteins in a single 10 ml. sample of cerebrospinal fluid or serum. The proteins were precipitated and delipidized with a methylal:methanol mixture, free cholesterol precipitated from the lipids with "natigine" and assayed by the Liebermann-Burchard method, cholesterol esters and lipid phosphorus in the mother liquor separated by distribution between petrol ether and aqueous alcohol, followed by colorimetric assay of each. In another paper Delsal (24) corrected a previous finding of his that boiling alcoholic potash or sodium ethylate attacked free cholesterol and attributed it to the use of impure material. Barac & Deltombe (25) have contributed yet one more study, to the many in the literature, on the reliability of the Liebermann-Burchard reaction especially when used to assay total cholesterol. The colour was estimated photometrically and was stable over a period of 5 min. with the technique used. The Beer-Lambert law was found to apply and the more intense colour developed with cholesterol esters than with the equivalent amount of free cholesterol was confirmed. Curiously, they found that total cholesterol in plasma can be determined without saponification, in contrast to mixtures of pure cholesterol and its esters, for which no explanation could be offered. Sols (26) estimated total cholesterol in serum by converting it all to ester with acetic anhydride and comparing this in the Liebermann-Burchard test with a cholesterol standard converted

into acetate under the same conditions. Wall & Kelley (27) have described both micro- and macromethods for assay of leaf sterols. Total sterols were determined by saponification, purification, and precipitation with digitonin, followed by either colorimetric (Liebermann-Burchard) or gravimetric assay. For the determination of free sterols, a chromatographic treatment replaced the saponification procedure. Discrepancies were found with certain species between the micro (colorimetric) and the macro (gravimetric) assays. This was found to be due to the presence of saturated sterols, which were assayed by separating them from the unsaturated sterols by partition between chloroform and the Liebermann-Burchard reagent. The colour reaction had to be standardized for each species with the sterol digitonides isolated from that species, since the different sterols involved gave different colour characteristics. Sterol esters were found to be generally absent or very small in amount. Whenever saturated sterols were present they made up 50 per cent or more of the total, which was always higher than when saturated sterols were absent. Hirsch (28) has suggested that a permanent standard for use in the Liebermann-Burchard reaction can be prepared from a mixture of nickel sulphate, ferric chloride, and sulphuric acid.

Until methods, such as chromatography, are available for the quantitative separation and isolation of pure lipid classes, studies of lipid distribution in blood and tissues must rely largely on calculations made from determinations of the products of hydrolysis. In such methods, of course, it is essential to take into account all the lipid classes known to exist in the tissue under examination, and the list of such lipids is steadily growing. Hack (29) has made use of the relative resistance of sphingomyelin to alkaline hydrolysis in estimating the phospholipids in human blood. When various lipids were hydrolyzed by 1 *N* KOH at 37°C. for 16 hr. sphingomyelin remained quite unattacked, but lecithin and cephalin were hydrolyzed with liberation of glycerophosphoric acid, acetal phosphatides yielded glycerophosphoric acid when the hydrolysate was acidified, and lipositol yielded phosphoric acid. Choline was also split from lecithin. Thus, assuming only lecithin, cephalin, and sphingomyelin to be present, the determination of choline and acid-soluble phosphorus obtained by alkaline hydrolysis enables the lecithin and cephalin to be calculated, and the difference be-

tween total phosphorus and phosphorus liberated by alkali gives a measure of the sphingomyelin. If other hydrolyzable lipids such as acetal phosphatides are present, they will be estimated as cephalin, a fate which in the past has befallen so many of the recently discovered lipids. Sinclair (30, 31) has compared the values for the lecithin, cephalin, and sphingomyelin content of serum as estimated (a) by the choline-phosphorus and nitrogen-phosphorus ratios, and (b) by the amino nitrogen and nondiffusible phosphorus content after differential hydrolysis by essentially the procedure of Hack (29), with reasonably good agreement. Compounds such as phosphatidyl serine would be estimated as cephalin, and phosphatidic acids, if present, would cause trouble in both procedures. The results suggested, fortunately, that "cephalin" only made up a small proportion of the total lipids in all but one (turkey) of the sera studied. Sinclair also noted that the crude acetone-insoluble lipids of serum were heavily contaminated by nitrogen-rich, diffusible impurities, which in some cases apparently included choline.

The acetal phosphatides are a group of lipids of widespread occurrence which have always proved difficult to estimate quantitatively, and which almost certainly on many occasions have been responsible for errors in the determination of other lipids. The need for a reliable method of estimation of these lipids is further stressed by the work of Ehrlich *et al.* (32), who showed that the fuchsin test, which forms the basis of all methods yet evolved, is seriously inhibited by the presence of other lipids with surface-active properties. The effect could be suppressed to a large extent by reducing the water content of the medium by a high concentration of acetic acid.

There are several papers of general interest in connexion with lipid analysis. Frampton *et al.* (33) confirmed the value of perchloric acid in the ashing of lipids for phosphorus determination. Sperry & Brand (34) have studied the use of various hydrolytic agents for sphingomyelin in the microdetermination of choline in brain lipids and found the best to be a half-saturated solution of barium hydroxide. A paper by Thannhauser (35) provides a useful summary of the methods of determination in serum of cholesterol, cholesterol esters, neutral fat, phosphatides, cerebrosides, and total fatty acids. Their clinical significance is also discussed and the values in normal and pathological specimens tabulated. Bloor

(36) has extended his oxidative method for the estimation of small amounts of fatty acids or cholesterol to a colorimetric procedure. Hsiao (37) has described a simple and rapid micromethod for the extraction of lipids from tissues. Enzyme hydrolysis may well prove to be a valuable tool in the study of the structure of certain lipids. In this connexion the discovery by Hanahan & Chaikoff (38) of an enzyme (in carrots) which is specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping is of interest, as is also the discovery by Fairbairn (39) of an enzyme in *Penicillium notatum* which can split off the saturated fatty acid from lyso phosphatides to leave the fatty acid-free skeleton.

Structure of lipids.—The steady advances in our knowledge of lipid structure made during the last few years have been maintained during the period of review. These advances are due in no small measure to more meticulous technique, in which both mother liquors and the crystals obtained therefrom are given equal consideration, and in which discrepancies between analytical and theoretical values are followed up instead of being merely noted and ignored.

The preliminary observation of Chargaff *et al.* (19) of a hitherto unidentified nitrogenous derivative in crude phosphatide fractions, as revealed by chromatography, has already been described. Their observation by the same technique that only galactose could be found as the carbohydrate in cerebrosides from normal tissue is in agreement with the work of Hsien-Gieh *et al.* (40), who confirmed their chemical and physical findings by fermentation studies.

The group of lipids incorporating sphingosine in their structure is a singularly difficult one to study. Carter *et al.* (41 to 44) have published a valuable series of papers dealing specifically with this group, for which they propose the name "sphingolipides" (41). In the second paper they reported the presence of dihydrosphingosine in the cerebrosides of brain and spinal chord. By an elegant series of studies (43) they confirmed the structure of sphingosine as $\text{CH}_3(\text{CH}_2)_{12}\text{CH}=\text{CH}\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2(\text{OH})$, and in the fourth paper they reported studies directed towards the synthesis of dihydrosphingosine and its isomers. These authors emphasised the occurrence of the dihydrosphingosine in the cerebrosides but did not rule out its possible simultaneous presence in the sphingomyelin fraction. Thannhauser & Boncoddio (45) applied

the observations of Carter *et al.* in their own studies of sphingomyelin and found that this group, from both brain and spleen, contained both sphingosine and dihydrosphingosine. In view of their discovery of dipalmityl lecithin as a gross contaminant of the sphingomyelin of earlier work, they reinvestigated the fatty acids of this group, as freed from hydrolecithin, and found that brain sphingomyelin contained lignoceric, nervonic, and stearic acids, in the approximate ratio of 1:2:2, and no palmitic acid, whereas spleen sphingomyelin contained only palmitic and lignoceric acids, in about equal proportions, as they had shown in earlier work to be true for lung sphingomyelin.

A considerable amount of work has been reported on the chemistry of other types of phosphatide. Thannhauser & Boncoddio (46) have followed up their earlier identification and isolation of a fully saturated lecithin (hydrolecithin) from the sphingomyelin fraction of beef lung by isolating the same dipalmityl compound from beef brain and spleen sphingomyelin fractions, of which it was found to make up about 25 and from 25 to 40 per cent respectively, illustrating how grossly these fractions were contaminated when separated by the usual procedures. Folch (47) has continued his studies on phosphatidyl serine and in addition to describing a method of preparation of products of at least 92 per cent purity has produced evidence that this lipid is the di-fatty acid ester of glyceryl-phosphoryl serine, the latter being united to the phosphoric radical through its hydroxyl group, leaving the amino group free. In the particular preparation studied, the fatty acids consisted of equal parts of stearic and oleic acids, and when isolated with neutral solvents the lipid was obtained as a mixture of sodium and potassium salts. Overman & Wright (48) isolated an inositol phosphatide from the total phosphatide fraction of the thromboplastin of lung tissue. This appeared to be identical with the inositol lipid from soy beans and from brain. Inositol phosphatide from all three sources was found to inhibit the action of thromboplastin. Hana-han & Chaikoff (49) have continued their work (already alluded to) on enzymes which can split off the nitrogenous base from phosphatides to give a phosphatidic acid. In addition to carrots, they found such an enzyme to be present in cabbage leaves and showed that the earlier finding of large proportions of phosphatidic acids might well result from enzyme action under the conditions of ex-

traction employed. This work emphasises a danger inherent in all work on lipids: the extreme lability of many of them, and possible post-mortem degradation by tissue enzymes. Fleury (50) followed the course of hydrolysis of egg lecithin with dilute aqueous potassium hydroxide at 35°C. Choline and fatty acids were liberated but, in contrast to hydrolysis with boiling alcoholic potash or cold barium hydroxide solutions, glycerophosphoric acid could not be identified. Instead, a "glycerophosphatogen" was obtained, from which glycerophosphoric acid could be slowly obtained by the action of calcium, barium, or lead salts. The phosphatogen was not obtained pure, but from its properties it was assumed to be a glyceryl derivative of pyrophosphoric acid. If it is not an artefact, and the mild hydrolytic conditions should be emphasised, then egg lecithin must be considered as a macromolecule. Desnuelle & Molines (51) have found that the strongly polar groups of the phosphatides cause them to become concentrated at a glyceride-air interface, as well as at a glyceride-water interface.

There has been a long history of controversy over the existence of α - and β - forms of the phosphatides, corresponding to α - and β - positions of the phosphoric acid radical. Baer & Kates (52) have made detailed studies of the hydrolysis of pure L- α -glycerylphosphoryl choline and shown that changes in optical activity could not be attributed solely to an α - β migration, but that this migration was reversible, leading to the production of DL- α -glycerophosphoric acid. The equilibrium ratios of α - and β -glycerophosphoric acids were a function of the pH, the α -form predominating in acid and the β -form in alkaline hydrolysis. These authors considered the possible series of reactions which might occur in acid hydrolysis and found that the experimentally determined reaction velocities as compared with the rates of change in optical activity necessitated the view that the greater part of the migration must have taken place while the choline ester was still intact, presumably via a cyclic ortho ester. They pointed out that α -phosphatides undoubtedly occur in nature, since optical activity can be demonstrated. Furthermore, when α -glycerophosphoric acid was isolated by enzyme action or by glycolysis it possessed the full optical activity of the pure synthetic compound. On the other hand, there is as yet no valid evidence of the natural occurrence of β -phosphatides, since all this evidence rests on the isolation of β -glycerophos-

phoric acid after chemical hydrolysis. The authors are studying enzymatic hydrolysis of synthetic α -lecithins of known constitution in the hope of developing an unequivocal procedure for the elucidation of the configuration of the naturally-occurring phosphatides.

There has been further work on the complex lipids of the tubercle bacillus. De Sütö-Nagy & Anderson (53, 54) have found that every lot of bacilli grown on artificial media elaborated different polysaccharides and different organic phosphoric acid derivatives. Thus, the phosphatides of one lot gave, on mild alkaline hydrolysis, inositol monophosphoric acid, glycerophosphoric acid, and a glycoside containing phosphorus from which both inositol and mannose were obtained. Another lot gave glycerophosphoric acid, inositol glycerol diphosphoric acid, and a glycoside which yielded glycerophosphoric acid, inositol, and mannose. These phosphatides contained about 70 per cent of fatty acids, including tuberculostearic acid and a small amount of some higher dextrorotatory liquid saturated acid. Polgar (55) has introduced a new technique for the separation of the fatty acids of the tubercle bacillus lipids by converting them into acetol esters, treating these with agents for ketones, such as semicarbazide or 2,4-dinitrophenolsemicarbazide, and fractionally crystallizing these derivatives. He isolated a C_{19} acid resembling tuberculostearic acid, but having a melting point some 12 degrees lower, and from a mixture of higher acids he isolated three dextrorotatory acids. The first two appeared to contain four terminal methyl groups, and the third, three. The presence of an unsaturated branch chain acid of high molecular weight was indicated by the isolation of its oxidation product. Cason & Prout (56) have considered the structure of phthioic acid in the light of its optical rotation, which they were unable to reconcile with the suggestion of Polgar & Robinson (57) that it is 3,13,19-trimethyltricosanoic acid. They considered that the absence of rings and quaternary carbon atoms had hardly been demonstrated.

Cornforth & Robinson (58) have discussed the possibilities of a total synthesis of cholesterol and progress to date. Weitkamp *et al.* (59) have examined the free fatty acids excreted by the sebaceous glands of the adult human scalp, which have important fungistatic properties, and which contain predominantly normal saturated and unsaturated fatty acids ranging from C_7 to C_{22} .

Both saturated and unsaturated acids containing an odd number of carbon atoms were found. The double bond in the unsaturated acids was typically in the 6,7-position, although some 8,9- and other isomers were present.

Lipid-protein complexes.—The subject of protein chemistry forms a separate section in this volume and it is difficult to decide how far work on lipid-protein complexes should be discussed in the present chapter. The following papers have been examined from the viewpoint of the lipid chemist and reference should be made to the chapter on protein chemistry for further consideration.

It is natural that most work on lipoproteins has so far been carried out on blood serum, since the complexes are present in soluble form and their separation and isolation is thereby simplified. Tayeau *et al.* (60) have investigated the effect of proteolytic enzymes, pepsin, trypsin, and papain, on the lipo-proteins of horse serum and found that there was hardly any "lipophanerosis" (freeing of lipids, rendering them directly extractable with ether). There was also evidence of less ready attack by the enzymes on the lipoproteins than on the free proteins, since the bound lipid content of the residual protein increased. It was suggested that even when lipoproteins were attacked, the lipid-protein linkages were left intact, leading to the formation of lipid-peptide complexes in which the lipid was still not extractable by ether. In a second paper, Tayeau & Breton (61) confirmed the greater resistance to proteolytic enzymes of lipoproteins as compared with free proteins, by testing serum before and after delipidization. In part three of the series, Tayeau & Breton (62) demonstrated the actual presence of lipid-peptide complexes by fractionation of trypsin digests of serum with trichloroacetic acid and phosphotungstic acid. These contained about one sixth of their weight of lipids, and the polypeptide portion was of relatively low molecular weight, with a total nitrogen: amino nitrogen ratio of 4.0 to 6.0. The lipids were very firmly bound and required the action of boiling alcohol in a strongly acid medium to liberate them. A summary of this work was reported earlier (63).

Blanquet & Tayeau (64) showed that the lipid-protein complexes in serum significantly increased the buffering power of blood to changes in surface tension induced by such substances as

bile salts. The lipoproteins also had a slight direct effect in raising the surface tension, but this was far less marked than their restraining influence on the effect of bile salts, as shown by comparative experiments on serum before and after delipidization by the Hardy-Gardiner technique.

There is continuing study of artificial lipid-protein systems as an aid in elucidating the structure of the naturally occurring complexes. Harris *et al.* (65) have continued their series of researches on the adsorption of paraffin chain salts by proteins. Experiments with deaminated gelatin provided further evidence that the linkages between normal gelatin and dodecyl sulphate ions are of two types, depending on the pH at which the complex is formed. At pH values of 5.5, for instance, the authors envisaged linkage by (a) adsorption of the dodecyl sulphate ions at the positively charged groups of the basic side chains of the protein, and (b) adsorption of the paraffin derivative at the backbone imide nitrogens of the protein, the link being of the ion-dipole type made possible by resonance of the keto-imide groups. At pH values of 2.5, only the former type of linkage was considered to operate, since the resonance required in the second type is known to be inhibited by hydrogen ions. Deaminated gelatin at pH 2.5 retained hardly any capacity to form a complex with dodecyl sulphate ions, but at pH 5.5 it could still do so, although the complexes were more soluble than those formed from intact gelatin. Partially hydrolysed gelatins were still able to form complexes at both pH values, which seems of interest in connexion with Tayeau & Breton's work on lipid-peptide complexes. In a following paper, Pankhurst & Smith (66) described some of the chemical and physical properties of dodecyl sulphate-gelatin complexes. Complexes can be prepared at pH 5.5 containing dodecyl sulphate ions equivalent to 93 per cent of the total nitrogen of the protein, and when these are completely dried they become insoluble in water but are completely soluble in organic solvents. The authors postulated that during drying there was irreversible formation of a covalent linkage, and they suggested a possible mechanism involving adsorption of hydrogen ions and elimination of water, with production of alkoxy-sulphonamide derivatives at the basic side chain nitrogen atoms. This work emphasises the possibility of change in the mode of

linkage of lipids and proteins when the complexes are dried, moistened, or otherwise subjected to changed conditions, a point which is further brought out in the work of Olcott & Mecham, to be discussed later (see p. 111).

Dervichian & Magnant (67) have also studied the addition of alkyl sulphates to gelatin, as well as to serum proteins. They found that sodium decyl sulphate, and lower members, gave neither precipitates nor coacervates—a coacervate being defined as a liquid phase in equilibrium with the mother liquor. With gelatin in acid solution, C_{12} , C_{14} , and C_{16} alkyl sulphates gave precipitates, which were transformed into coacervates if the temperature was raised above 30°C . With horse serum albumin or pseudoglobulin, however, the precipitates were not changed into coacervates at temperatures above 30°C . With excess of reagent, re-solution took place, suggesting the formation of a soluble complex. [Pankhurst & Smith (66) also noted this phenomenon, which they attributed to the adsorption of a second layer of dodecyl sulphate ions with their hydrophilic groups directed outwards.] Coacervates could, however, be obtained from serum pseudoglobulin by the adsorption of myristyl choline or stearyl choline. Again excess of reagent resulted in re-solution. In a second paper (68), the authors discussed the nature of coacervates and their formation.

In general much less is known about the lipid-protein complexes of plants than of animals, and three papers on this subject are, therefore, all the more welcome. Antener & Högl (69) in a series of studies of the extractability of the lipids of wheat germ with various solvents under a variety of conditions obtained evidence that although the fat and the unsaponifiable matter were largely "free," much of the phosphatides was "bound." Part of the bound lipids were extractable with such solvents as trichlorethylene, which could, therefore, split the lipid-protein links at least in part. However, if the wheat germ was first given a short treatment with boiling alcohol, followed by extraction with petrol ether (Terrier's method), still more phospholipid was extracted, and this was the only extract which also contained carbohydrate. The carbohydrate-containing extract gave a clear solution in petrol ether. The authors postulated that in wheat germ the phosphatides were largely linked on the one hand to pro-

tein and on the other to carbohydrate. Solvents such as trichloroethylene split the lipid-carbohydrate link and partly split the lipid-protein link. Short-time treatment with boiling alcohol, however, could split quantitatively the lipid-protein link and leave the lipid-carbohydrate link intact. In a second paper (70) the same workers reported more detailed studies of the phosphatide-carbohydrate complex. The carbohydrate was identified as sucrose. The phosphatides apparently consisted of a mixture of lecithin, cephalin, and magnesium phosphatide. The evidence that the phosphatides and carbohydrate were really linked in a complex, and not just loosely associated by simple adsorption, may be summarized: (a) the ratio of sugar to phosphorus remained approximately constant in a series of preparations; (b) fractionation experiments which led to enrichment in phosphorus also led to enrichment in sugar; (c) the sugar could not be split off by shaking with water although it could be so split from artificially-prepared lecithin-sugar complexes which were likewise completely soluble in petrol ether. The most concentrated preparation contained 49 per cent phosphatides and 43.3 per cent sucrose, the remaining 7.7 per cent being probably magnesium phosphatide or some similar substance. The mode of linkage of the sugar and lipid could not be elucidated. An ester linkage seemed to be ruled out, since after splitting off the sugar with aqueous alcohol there was no increase in the acid value. The complex was not destroyed by heating the germ to 110°C. (the caramelization point) before extraction, but it was destroyed, presumably by enzyme action, when the germ was moistened and incubated at room temperature.

Olcott & Mecham (71) have studied the extractability of the lipids of wheat gluten. About 70 per cent of the lipids of flour could be extracted with ether. When the flour was wetted without doughing, and then dried by lyophilization, only 40 per cent was extracted. After doughing the percentage was reduced to 6. If flour lipids were added to flour, which was then doughed, the added lipids were largely "bound," up to three times the amount present in the original flour. Phosphatides were bound preferentially to the nonphosphatide portion of the lipids. Similarly, half of the ether-soluble lipids, but almost 90 per cent of the ether-soluble phosphorus, became bound during the wetting of the flour. They

confirmed earlier observations that the lipids largely accompanied the gluten fraction. On fractionation into glutenin and gliadin the lipids accompanied the former, which contained about 20 per cent of lipids, nearly all bound. Glutenin, therefore, as it occurs in gluten but not necessarily as it occurs in the wheat grain, was considered to be a lipoprotein, which should be called "lipoglutenin," the name "glutenin" being reserved for the lipid-freed protein.

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CHEMISTRY OF AMINO ACIDS AND PROTEINS¹

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PROTEIN ISOLATION, PURITY, AND CHARACTERIZATION

A continuing main interest in protein research has been the extent to which protein species are distinguishable as chemical individuals of reproducible composition, structure, and physical, chemical, and biological properties. Progress rests on discovery and refinement of means of fractionation and of tests of identity. Practically, purity is referred to intended uses and analytical methods and the relation of these to the interconversion of forms in equilibrium (1, 2), such as solvated or dissociated species. The reliable, thermodynamic uniform solubility method (3, 4) is limited in demonstrating trace impurities unless these can be estimated by specific properties such as biological activity or radioactivity. The similar criterion, constant partition coefficient between immiscible liquids (5, 6), is also the basis for separations and tests of identity. Chromatographic methods (292), especially applicable to mixtures containing amino acids, are based upon either adsorption or partition; both factors may operate together (7, 8). Kinetic criteria (9): diffusion (10 to 14), ultracentrifugation (15), and particularly electrophoresis with critical analysis of the form of the concentration gradient (16, 17), are so refined that no standard protein tested can be said to be unequivocally homogeneous. McDonald (18) has recently freed ribonuclease, the best of the group studied (16, 17), from most of the proteinase activity characteristic of earlier crystalline preparations.

Mark (15) has reviewed the problem of purity with special reference to synthetic high polymers. Methods at least potentially useful in protein research that have been applied to synthetic polymers include the determination of turbidity in dilute solution as a function of precipitant concentration, to discriminate components; the precise analysis of light scattering, as by Doty (19) and Zimm (289), as a sensitive means of molecular characteriza-

¹ This review covers the period from December, 1947 to December, 1948.

tion; and the use, in accordance with a suggestion of Debye, of diffusion in a thermal gradient for separating high molecular species according to molecular weight. Cann *et al.* (20) have developed fractionation by electrophoresis (288) of proteins which are then stratified by density gradients. Since it is possible to use very slight differences in electrical mobility to separate inorganic ions, applying the countercurrent principle (21 to 24, 290), it may also be practicable to separate even closely similar proteins or amino acids by this method. Edsall & Foster (25) continue to apply flow birefringence (291) to the study of molecular length and homogeneity; by this method Fredericq (26) has shown molecular changes in egg albumin during denaturation. Oudin's (27) potentially valuable immunological test of homogeneity consists of layering an antigen solution over a gel containing antibodies produced by immunization either toward that antigen fraction or toward a natural mixture including it. One or more precipitation zones form in the gel, and under suitable conditions a one to one correspondence can be shown between these and the antigens in the test solution.

Crystallized proteins.—Crystallinity, a notoriously unreliable criterion, is a convenient rule-of-thumb index of protein purity. Its main value lies in the possibility of deducing molecular size and shape from the x-ray study of crystalline material. Palmer *et al.* (28) report lysozyme especially suited to such study because its salts, the chloride, bromide, and iodide, are easily crystallized, and because of its relatively low molecular weight, $13,900 \pm 600$ for the dry, chloride-free protein. Grown in acid solution, the crystals are tetragonal, in alkaline solution, orthorhombic (29).

Though usually artificial, crystallized proteins sometimes occur in nature. Crystalline cytoplasmic inclusions occur in silkworms infected with the polyhedral virus. Bergold's kinetic studies (30) of crystals formed in three species show slightly, perhaps significantly, differing constants. The virus itself was not crystallized, though a preparation from one species was centrifugally single-boundaried; it was much higher in molecular weight, although immunologically similar to the noninfectious crystals. Rich (31) attributes similar crystalline inclusions formed in *Phaseolus* after virus 2 infection to interaction of the virus and nucleoli of the cells.

From beef pancreas, Kunitz (32) has isolated crystalline desoxyribonuclease. Fruton (33) has confirmed the specificity of the crystalline pancreatic zymogen of Laskowski *et al.* (34), activated, to be that of a chymotrypsin, now named chymotrypsin(ogen) B.

Crystalline α -amylases, besides that from hog pancreas (35), have been prepared from bacteria (36) and from human pancreas (293) by Meyer *et al.*, and from malted barley by Schwimmer & Balls (37). Danielsson (38) has reported the physical constants of a malt amylase preparation (39) with both alpha and beta activity. Balls *et al.* (294) described a crystalline sweet potato β -amylase. In a continuing study, Rossi-Fanelli (40) found the crystallographic constants of human met- and oxymyoglobin, with chemical analyses, to confirm the difference from hemoglobin. Keilin & Schmid (41) described in detail the crystallization of met- and carboxymyoglobin from a whale (species not reported). Bowen described crystalline myoglobin from horse heart (317).

By prior determination of eight solubility minima in the curve of solubility as a function of pH at constant ionic strength, Distèche (42) has isolated three different crystalline preparations from rabbit muscle extract. From muscle extracts the Coris and their associates have prepared crystalline aldolase (295) and D-glyceraldehyde-3-phosphate dehydrogenase (43). Bonnichsen & Wassén (296) isolated from horse liver a crystalline alcohol dehydrogenase different from that of yeast.

Systematic measurement of incremental precipitation is of special value in finding the best conditions for separating components. A recent study of this sort is that of Baudouin *et al.* (44, 45) on salt fractionation of serum proteins in extremely dilute solution as a function of temperature and pH. Tustanovskii (46) has isolated crystalline protein in 2.7 per cent yield by dialysis of oxalate or citrate extracts of rat or rabbit skin.

Following extensive x-ray study of hydration of horse hemoglobin (47), Perutz (48) has deduced that, although red cells are not birefringent, random distribution of the hemoglobin is unlikely because the computed molecular clearances are only just enough for free rotation. Perutz & Weisz (49) find human carboxyhemoglobin more complex in crystal structure than horse hemoglobin. The unit cell of the human protein has four almost isodiametric molecules differently oriented towards the crystal axes. Cosslett & Markham (50) observed with the electron microscope a regular, diamond-lattice type array of the molecules of turnip yellow mosaic virus. From the lattice dimensions a slightly smaller diameter, 19.5 $m\mu$, is computed than that found by measurement of individual spheres.

Though crystalline insulin has been known for some time, uni-

form preparations of constant solubility have been hard to attain. Lens (51) now reports suitable conditions for recrystallization and for measurement of the solubility curve. It is necessary to minimize time of contact with media more acid than about pH 4.2. This requisite is illumined by Gutfreund's continued study (52) of the dependence of the insulin kinetic units upon physical conditions. He notes a maximum molecular weight, 47,000 to 48,000, of centrifugally single-boundaried samples at pH 7 to 7.5 in concentrations of 0.4 to 0.9 per cent. Dissociation appeared on dilution, or at higher or lower pH, to a minimum molecular weight of 12,000 at pH 2 to 3.

Electrophoresis and protein purity.—Electrophoresis is more widely useful as an analytical control for the resolution of complex natural mixtures than a critical test of purity because a single comparison under given conditions can show the relation of a fraction to the starting material, but an extended series of analyses under widely varied conditions, with special attention paid to the form of the moving boundary (16), is needed to establish electrophoretic uniformity with reasonable surety. Hoch (53) discusses some less usual aspects: the changes in field strength and mobility at boundaries under given conditions. The average net charge is found from the rate of change of mobility with concentration; the effect of components of similar mobility upon their apparent concentration relationship is also related to this rate of change. It is stressed that examination for purity should be made also at low concentrations where interference is least, in exploratory experiments over the whole concentration range allowed by diffusion. However with proteins such as insulin (52), the possibility of dissociation with dilution should be considered. Alberty (54, 55) has begun an extended general review of electrophoretic technique and theory.

Kunitz & McDonald (56) have prepared crystalline ricin of constant toxicity, single-boundaried in ultracentrifugation and electrophoresis. A phase rule study showed the crystals to be a solid solution of two or more components. Lamanna & Doak (57), by a milder isolation procedure, have made a new crystalline preparation of the *Clostridium botulinum* Type A toxin with slightly revised (58) physical constants. The toxin was electrophoretically single-boundaried under a given set of experimental conditions. The toxicity was variable; solubility and sedimentation showed other evidence of heterogeneity, attributed to the extreme lability of the

toxin and, under certain conditions, nontoxic dimerization (58). On the other hand, protein fractions of high biological activity often appear electrophoretically complex. Fishman *et al.* (59) have prepared a crystalline protein of high growth hormone activity by alcohol fractionation of a lime water extract of beef anterior pituitary; one preparation was electrophoretically and centrifugally single-boundaried. Kazal *et al.* (60) have crystallized a pancreatic trypsin inhibitor and anticoagulant different in properties from that of Kunitz & Northrup; three electrophoretic components were present, at least two of them active.

Of proteins not crystallized, Keilin & Hartree (61) record the enzymatic properties and absorption spectrum of the flavoprotein glucose oxidase (notatin, or penicillin B) (62). Their electrophoretic study and measurements of sedimentation and diffusion by Cecil & Ogston (63) indicate 80 to 90 per cent purity. Pillemer *et al.* (64) have isolated diphtheria toxoid of constant solubility. Charlwood (65) finds diphtheria toxoid preparations to have two or three components, activity being associated with the slower (less acid). The major components have maxima in their pH-mobility curves near pH 7 to 8, measured in phosphate buffer of ionic strength 0.1, the mobility being slightly less in the phosphate buffer of highest pH (8.0) and in borate. Such an effect may be due to specific anion binding, decreasing with increasing pH as the intrinsic charge of the protein increases.

The preparation of tyrosinases from *Psalliotia campestris* has been reported upon (66). An electrophoretically single-boundaried catecholase fraction, dehydrogenating ortho dihydric phenols, was distinguished from a cresolase fraction, only 70 to 80 per cent pure electrophoretically, which oxidizes monohydric phenols aerobically through ortho hydroxylation. The copper contents were such that the cresolase fraction could have contained copper-free components associated with 14 per cent of the copper-containing catecholase.

Jameson (67) has made interesting comparisons of electrophoretic patterns developed from boundaries at which plasma or serum was layered at different finite concentrations. The proportion of faster moving components appeared to increase with dilution, at the expense of the γ -globulin. In such an experiment it should be recognized that the presence of a component on both sides of an electrophoretic boundary may cause the observed mobility and

concentration gradient of a boundary to be related in no simple way to the true mobility and concentration of the particular component.

Cohn & Wolfson (68, 69), Bock (70), Kibrick & Blonstein (278), and Jager & Nickerson (297) analyze clinical methods for separating albumins and globulins. Avery & Munro (298) prefer potassium phosphate precipitation for the preparation of electrophoretically uniform, completely coagulable human fibrinogen.

Staub & Rimington (71) isolated a seromucoid fraction from ox blood which contained 70 per cent of the nitrogen and 40 per cent of the bound hexose remaining after removal of heat coagulable proteins. The preparation had constant solubility in the presence of excess solid, constant hexose/nitrogen ratio, and was electrophoretically single-boundaried in a single analysis. Guest *et al.* (72) have described a euglobulin plasma fraction, vascularin, stable to heat and reduction, which has pressor activity as a result of smooth muscle stimulation, and also causes leucopenia and decreases cardiac output. Goldberg & Hass (73) have separated antinvasin I into two components by salt-alcohol fractionation of hog or human serum. Holmberg & Laurell (74) relate serum histaminolytic activity to a copper-containing globulin.

Nichol & Deutsch (75) have prepared γ -globulins for comparative study by salt-alcohol fractionation of the sera of seven animals, including man. In a more intensive study of bovine γ -globulin, Hess & Deutsch (76) prepared a fraction homogeneous in sedimentation and diffusion, but spreading in electrophoresis. By electrophoresis, Roubert (77) showed horse γ -globulin to have 5 to 12 components. Basset & Paille (78) reviewed the relations of antibodies to the protein fractions of antimicrobial sera. Harms (79) described in detail the large scale purification of antitoxins from horse sera, using carefully controlled older methods: peptic digestion, heat denaturation, and salt fractionation without alcohol. Koprowski *et al.* (80) studied the sera of rabbits and chickens immunized against the viruses of Japanese B encephalitis, Venezuela horse encephalomyelitis, and western horse encephalomyelitis. The γ -globulin fraction contained material active against the first, while antibodies against the latter two occurred as both β - and γ -globulins. By salt-alcohol fractionation of horse sera, normal or variously hyperimmunized, Deutsch & Nichol (302) find that antibodies, although of widely distributed electrophoretic mobilities

according to the antigen, generally precipitate as γ_2 - or water-soluble γ_1 -globulins.

The heterogeneity of β -lactoglobulin.—Recent studies of β -lactoglobulin show especially well the problems met in isolating a pure protein. Readily crystallized (81), this popular model has been intensively, but not conclusively, analyzed chemically, for example, by Brand *et al.* (82) and by Tristram (83). Bull & Currie (84) noted its susceptibility to injury on slight exposure to alkali in the usual isolation, and by osmotic study of preparations recrystallized without use of alkali found a molecular weight of 35,000, distinctly lower than earlier estimates by, for example, Gutfreund (85). By x-ray measurements, Senti & Warner (86) estimated a molecular weight of 35,500 for similar material. Bull (87) has observed a dissociation to units averaging half this weight in the course of film pressure studies on 10 to 40 per cent ammonium sulfate. Even after primary denaturation in urea, a large proportion was recovered in crystalline form by Jacobsen & Christensen (88).

Li's finding (89) that a standard preparation was electrophoretically triple-boundaried at pH's 4.8 and 6.5, although single at 5.3 and 5.6, and Smith's observation (90) of the unusual variation of the mobility and apparent proportion of β -lactoglobulin in whey analyzed at various protein concentrations, with resolution of 9 to 14 per cent of a minor component, support the earlier conclusions of Grönwall (91) based on solubility studies. Extending these data, McMeekin *et al.* (92) have prepared a series of crystalline fractions of progressively increasing solubility, electrophoretically single and identical at pH 8.3, but showing increasing amounts of a never negligible second component at pH 4.8. Both McMeekin *et al.* and Bain & Deutsch (93) have attempted low temperature fractionation in alcohol. The latter also noted a single electrophoretic boundary at pH 8.6, but complexity at lower pH's. This change with pH appeared to be reversible. The possibility of aggregation in alkaline solutions was not assessed. Alberty *et al.* (17) found material fractionated in alcohol, having less than 7 per cent electrophoretically resolvable impurity at pH 8.6, to give a single boundary, nearly, but not critically ideal in form near the isoelectric point at ionic strength 0.1. By reducing the ionic strength to 0.01 they were able to resolve a minor component amounting to 30 per cent of the whole. At the higher ionic strength, careful measure-

ment of reversible boundary spreading showed heterogeneity comparable to that of ovalbumin, known to have 25 per cent of electrophoretically resolvable minor components. Even after electrophoretic resolution the main component showed measurable reversible boundary spreading.

From the evidence cited, we may consider three sources of in-

TABLE I
ELECTROPHORETIC EVIDENCE OF THE NATURE OF IMPURITIES ASSOCIATED WITH β -LACTOGLOBULIN

Author	Conc.*	pH	Anion†	Ionic strength	Mobilities of components (percentages in parentheses)‡		
Li (89)	1.5	4.8	Ac	0.1	2.3 (68)	1.9 (22)	1.2 (10)
	1.5	5.3	Ac	0.1	-1.4		
	1.5	5.6	Ac	0.1	-2.5		
	1.5	6.5	Ph	0.1	-5.6 (48)	-4.5 (27)	-5.2 (25)
Alberty,		5.22	Ac, Cl	0.1	nil (100)		
Anderson &		5.31	Ac	0.01	nil (70)	1.3 (30)	
Williams (17)		8.6	V	0.1	nil (93 or more)		
McMeekin	1.0	8.3			"usual"		
<i>et al.</i> (92)	1.0	4.8	Ac	0.1	2.2 (72)	1.4 (28)	to
	1.0	4.8	Ac	0.1	3.2 (41)	1.5 (59)	
Smith (90)	Mobility of β -lactoglobulin in whey						
	1.85	8.6	V	0.1	-5.0 (58)		
	0.5	8.6	V	0.1	-5.6 (48)		
	Mobilities of the immune globulins of whey						
		4.8 univalent				1.4	1.0
		5.3 univalent				0.9	0.5
		5.6 univalent				0.6	0
		6.5 univalent				-0.5	-1.2

* Concentrations are in grams per hundred cc.

† Anions referred to are acetate (Ac), phosphate (Ph), chloride (Cl), and diethylbarbiturate (V).

‡ Mobilities are in units of 10^{-5} cm.² volt⁻¹ sec.⁻¹

homogeneity. One is that preparations may contain varying amounts of distinctly different proteins also found in milk. Smith (90) and Deutsch (94) have explored the properties of whey proteins. The electrophoretic data summarized in Table I suggest that the principal electrophoretically separable contaminants of the preparations studied by Li, Alberty, and McMeekin are one or more of the immune globulins characterized by Smith. Nonresolu-

tion of minor constituents near the isoelectric point may be due to complex formation, especially when their net charge is opposite to that of the main component. They may also be slow enough to be overlooked as part of the delta and epsilon boundaries. The one big discrepancy between Li's and Smith's data at pH 6.5 may be due to Li's use of a phosphate buffer, in which several proteins are known to have mobilities higher than in univalent electrolytes. Degradation products, aggregated, dissociated, or otherwise changed are other possible impurities. Finally, β -lactoglobulin proper, free of degradation products and kinetically discrete contaminants, may consist of a closely related group of molecular species with graded properties, discernible by methods such as Alberty's, but requiring added evidence to be distinguished from the preceding possibility.

INTERACTIONS IN PROTEIN SYSTEMS

Intermolecular forces and binding energy.—Interaction of proteins with ions or other molecules is well known to involve interplay of several kinds of attractive and repulsive forces. This generalization rests on many recent studies of protein interactions with inorganic or organic agents, including dyes, detergents, drugs, and other compounds of biological interest. These studies have used measurements of solubility, kinetics of biologically specific reactions, surface film pressure, acid-base titration, electrophoresis, ultrafiltration, absorption spectra, chemical analysis, and (95) equilibrium dialysis.

The slight changes in infrared absorption (301) attending combination of sodium dodecyl sulfate with various amino acids and model substances imply that combination in these instances is mainly electrostatic, hydrogen bonding, for example, being only slightly affected. Combination of ions with proteins under conditions where the charges are similar, as of methyl orange with serum albumin above its isoelectric point (96), is attributed to preponderance of van der Waal's forces lowering the potential energy of the protein-ion system despite unfavorable electrostatic conditions. In exploring structural features that affect interaction of sulfonamides with serum albumin, Klotz & Walker (97) by equilibrium dialysis found the free energy of binding dependent on the concentration of the drug anion as defined by its pK_a and the pH of the solution. Thus at pH 7.8, 2-sulfanilamido-5-chloropyridine, 5.7 times more ionized than sulfapyridine at this pH, is bound to serum albumin

with an energy 960 calories per mole greater; this is in agreement with the computed higher anion concentration ($960 \cong RT \ln 5.7$ at 25°). Though the effects of various van der Waal's forces, viz., dispersion, induction, polarization, and resonance, usually appear indirectly through changes in the acid dissociation constant, a direct effect of van der Waal's forces appears on comparing N-acetylsulfanilamide with N-benzoylsulfanilamide. The anion concentrations of these compounds are essentially equal at pH 7.6, yet the benzoyl compound has a binding energy with serum albumin about 760 calories more than the acetyl compound. This behavior evinces the greater effect of the phenyl group compared with methyl. Like differences were noted by Gilbert (98), in comparing the reaction of several dye anions with wool, and by Steinhardt (99), showing increasing affinity of wool for anions of increasing size.

Teresi & Luck (100) observed a similar increase in binding energy with molecular size, apparently due to dispersion forces, in equilibrium dialysis of serum albumin with several carboxylates and nitrophenolates. Phenylbutyrate is bound more strongly than phenylacetate. Cinnamate, though smaller than phenylbutyrate, is more firmly bound, probably because of the polarizing influence of the double bond. Increased binding energy resulting from introduction of nitro groups in phenolic compounds is attributed to the highly inductive properties of the electronegative nitro group as well as its contribution to molecular size.

Klotz & Urquhart (101) observed the free energies of binding of adenine, adenosine, and adenylic acid with serum albumin, respectively $-3,080$, $-3,370$, and $-3,910$ calories per mole, to be much lower than the values for binding of various dyes and sulfa drugs of similar size. This lower binding energy they proceed to ascribe to the more polar nature of these compounds, favoring their remaining with the solvent. Ortho substituted benzenecarboxylates or -sulfonates showed consistently higher affinities for serum albumin than did the para isomers if the ortho substituent were a hydrogen-donating group such as amino or hydroxyl. Such ortho groups are presumed to form an intramolecular, chelate, hydrogen-bond structure in preference to interaction with water. Para substituents are too far apart to chelate. Therefore, they become solvated and their interaction with protein is hindered.

An example of binding related indirectly to hydration is that of copper ions to serum albumin (102). This reaction occurs, despite

an unfavorable heat of reaction, because of an entropy increase probably due to liberation of bound water.

Serum albumin is often used for interaction studies because of its availability in relatively pure form and also because of its unusual power of binding various molecules and ions, a reflection, perhaps, of its biological role. The stabilizing effect of certain anions, caprylate, dodecylsulfate, acetyltryptophane, and mandelate, towards heat is of medical importance in sterilization (103). These anions also restrict the increased viscosity otherwise seen in urea solutions (104). Though caprylate and dodecylsulfate do appear to stabilize serum albumin against denaturation, other ions, specifically mandelate, probably merely prevent coagulation of the denatured protein (105). These ion interactions, especially with other proteins, are not at all fully explored. The serum globulins appear incapable of stabilization approaching the degree found with albumin. Klotz *et al.* (106) report interaction of hemocyanin with methyl orange with a binding energy only one-tenth that shown with serum albumin; the cationic dye chrysoidin is not measurably bound to hemocyanin, although its binding energy to serum albumin is only 1,000 calories less than that of methyl orange. Conversely, lysozyme binds no methyl orange (107).

Wright & Schomaker (108) assume combination of urea with diphtheria or staphylococcus antibodies with retention of their specific native configurations in interpreting the kinetics of urea inactivation. In each case the inactivation rate, measured by specific precipitation, showed first order behavior only with respect to initial antibody concentration; as the reaction proceeded, the rate fell off more rapidly than predicted by a first order law. Such kinetic behavior characterizes two competing reactions; in this instance one is the irreversible inactivation of antibody and the other the reversible combination of urea and antibody to form an active complex more stable than the original antibody. This active complex is supposed to be slowly inactivated through reversion to the original antibody or its kinetic equivalent.

Klotz *et al.* (109) have presented evidence for complex formation of urea with native protein more rapidly than urea-induced denaturation. A smaller interaction constant than those of ions such as methyl orange or the sulfa drugs characterizes interaction of urea with serum albumin. Because of this low affinity constant, urea is effective only in high concentration. In an environment of

several reactive species, combination with the protein depends on the relative affinities, but also on the relative concentrations. Thus six molar urea can displace methyl orange from serum albumin even though the dye has much the greater affinity. In general such reactions follow the principle of mass action.

Aten *et al.* (318) observed a marked decrease in specific optical rotation of serum albumin accompanying denaturation in 50 per cent urea. Removal of urea by dialysis led to recovery of material soluble in 5 per cent (or presumably more dilute) urea; this product had a specific optical rotation near that of the undenatured form in either water or 5 per cent urea.

Abundant evidence exists that ions such as various dyes, detergents, and thiocyanate (110) react at acid or basic sites, but influences more specific than acid or basic strength are involved, for example, in the interaction of protein sulfhydryl groups with heavy metals (111). Regarding the site and mechanism of interaction of cupric or ferrous ions in blood, Cohn (112) suggests competitive binding of these ions with siderophilin, a β_1 -pseudoglobulin in plasma fraction IV. Holmberg & Laurell (113) find that copper is readily removed from siderophilin by reaction with diethyldithiocarbamate or by specific replacement by iron. On the other hand copper is firmly bound to α -globulin, from which it is removed neither by iron nor by the carbamate. Schade *et al.* (114) noted a formation of salmon-pink ferrous complexes, in the presence of equivalent bicarbonate, with siderophilin or conalbumin. Similar absorption spectra are given by complexes of iron with aspergillid acid, hydroxylamine, or several cyclic hydroxamines. Fiala & Burke (115) propose a common, unproved mechanism for these examples of iron binding: formation of a specific co-ordination complex.

Structural influences on interaction.—Interacting molecules are understood to come to an equilibrium distance of minimum potential energy. Sufficiently close approach may be hindered by inert groups near the reactive groups. Unreactivity of some disulfide, thiol, and phenolic groups in native proteins is thought to be due to such structural features. Protein reactions such as those cited give insight to protein structure, for as Fuoss (116) says, the appropriate reagents function as "molecular scale explorers whose commission it is to study and report on the navigability and topography within the polymer maze."

Structural influences on ion interactions have been noted. Equilibrium dialysis allows independent estimation of the number of sites involved on the protein molecule as well as the binding energy. Marked differences occur with similar reagents. Teresi & Luck (100) find, for example, that serum albumin binds only six orthonitrophenolate ions, but 25 of its para relative. Again, seven phenoxyacetate or nine hippurate ions are bound, but a maximum of 25, corresponding to the number of basic groups of this protein, is attained with phenylacetate or phenylbutyrate. Although equilibrium is presumed, these differences might be laid to differences in reaction rates, with failure to reach true equilibrium. Even so, the differences in activation energies evince a barrier to interaction. In the cases of phenoxyacetate and hippurate, the barrier may be an effect of water bound by the polar substituents preventing sufficiently close approach to less accessible sites.

When an agent reacts nonspecifically with various similar groups, the effects of the different reactivities of these groups may be distinguished from reactivity differences due to the adjacent structure. Hallas-Møller (117) infers from polarographic data that the basic groups of insulin show characteristic differentiated activity depending upon their basic dissociation constants. Phenyl isocyanate reacts first with the histidine and lysine groups of insulin and then, with loss of activity, with the arginine residues.

Where reactions appear to be clearly structurally conditioned, groups near the surface of the molecule might be supposed to be more reactive than those buried inside. However, protection can be given a reactive group even on the surface. One chemical means to this end, able to suppress the characteristic properties of thiol and phenolic groups, is intramolecular hydrogen bond formation. Differences in binding energies of ortho and para substituted benzenecarboxylates, because of chelation, have been cited. In the same way, suitable groups on the protein surface may suffer suppression of reactivity through local steric effects as well as through solvation. Desnuelle & Casal (118) give an example of the opposite effect, increased reactivity due to the detailed local structure. Peptide bonds involving the amino groups of serine or threonine residues are more easily hydrolyzed by acid than others, supposedly because of intramolecular cyclization to unstable oxazolines.

Denatured proteins are often more reactive than native proteins. Klotz *et al.* (109), however, find native serum albumin the

more reactive toward methyl orange, just as Davis & Dubos (119) found it the more reactive toward oleic acid.

The reactivity of fibrous proteins is also affected by structure. Groups found in the crystalline portion of the fiber are known to be less reactive than those in amorphous regions, so that differential kinetic penetration by water or dyes, or acid hydrolysis can be used with fair success to estimate the degree of crystallinity of these materials, as confirmed by x-ray and birefringence measurements. Mellon *et al.* (120) studied the influence of crystal lattice forces on the interaction of the peptide bond with water; water absorption by a series of synthetic peptides showed the smaller, more perfectly crystalline, members to be unhydrated, more and more water being bound as the chain lengthens.

Hughes *et al.* (111) have characterized crystallizable complexes of serum albumin with mercury, iodine, and platinum chloride. McMeekin *et al.* (121) find β -lactoglobulin to crystallize with two molecules of dodecylbenzene sulfate.

Jirgensons (122), in studies with potato albumin, legumelin, legumin, and a modified, water-soluble legumin, has obtained further evidence of the appearance of thiol groups and increased viscosity on denaturation by pyridine, heat, or urea.

This review will not discuss the development of new methods for chemically modifying proteins with minimum change in the natural structure, except for a few examples showing the effect of structure on the reaction. Porter (123) finds Sanger's reagent, fluorodinitrobenzene, reactive with elimination of hydrogen fluoride at sites containing active hydrogen, to show different activity toward the native and denatured forms of several proteins. With native β -lactoglobulin, only 19 of the 31 lysine epsilon-amino groups react until the protein is denatured with acid, alcohol, heat, or guanidine; all are then reactive. With serum pseudoglobulin, 56 of the 80 epsilon-amino groups react before denaturation, afterwards all but two or three. Marked differences also appear among native proteins. Native bovine serum albumin has 57 of 59 epsilon-amino groups reactive, while native bovine and porcine β - and γ -globulins have only 40 to 48 of the 85 groups reactive.

Like certain acetylating agents, fluorophosphates can effect total inhibition of enzymes without affecting crystallizability. Diisopropylfluorophosphate (DFP) apparently specifically inhibits those enzymes having esterase activity (124). Jansen *et al.* (125) find esterase and proteinase activities of trypsin and chymotrypsin

equally inhibited by small amounts of DFP. Only 1.7 moles of DFP completely inhibit one mole of chymotrypsin, which remains inactive after recrystallization, dialysis, and lyophilization. The phosphorus moiety of radioactive DFP is bound by chymotrypsin quantitatively (126), but not by chymotrypsinogen, which remains potentially active. Therefore activation of the zymogen liberates not only groups necessary for enzyme activity but also those reactive with DFP.

Sumner (127) disputes the assumed correlation of enzyme activity with the native condition of the enzyme, reporting urease to be denatured by one or two days' treatment with 30 per cent alcohol and sodium chloride at room temperature. The "still highly active" product is "quite insoluble" in water or dilute phosphate buffer and gives a strong thiol test, but is more resistant to trypsin than is heat-denatured urease. The evidence seems still to permit the presence of native urease, adsorbed, for example, on the denatured material.

Most known enzyme inhibitors are small molecules, the reactions of which illuminate the basis for enzyme reactions and specificity. Protein inhibitors are also useful in studying the factors affecting protein interaction. Several proteins inhibiting trypsin have been isolated (60, 128, 132) or discriminated (299), and the chemistry of trypsin inactivation explored (300). Both the crystallizable inhibitor from soybeans (128) and ovomucoid (129) inhibit and combine with trypsin in equimolar ratio. Kunitz (130), noting a loss in amino nitrogen on combination of trypsin and soybean inhibitor, suggested reaction of the amino groups of the enzyme with carboxyl groups of the inhibitor. Activity studies of both proteins, chemically modified, leads Fraenkel-Conrat *et al.* (131) to infer that several kinds of reactive groups of ovomucoid are necessary for inhibition. Ionic attraction may play a part since, supporting Kunitz' idea, amino groups of trypsin and carboxyl groups of ovomucoid are both necessary for inhibition, although the amino groups of trypsin are not necessary for its enzyme activity. A highly active inhibitor, different physicochemically and analytically from both soybean and egg white inhibitors, has been prepared from lima beans (132).

Quantitative measurement of hapten inhibition of serological precipitation gives insight into the structural basis of biological specificity. Assuming an interacting region of the antibody complementary to the structure of the hapten, one may infer the struc-

ture of this region from the character of interacting haptens. Developing the program begun by Landsteiner, Pauling and his associates have extensively and minutely studied factors affecting antibody reaction. Pressman *et al.* (133), systematizing the inhibitory effects of more than 50 potential haptens on the precipitation, by its antiserum, of egg albumin coupled with *p*-azosuccinylate, show the first requirement for a hapten to be an electrically charged carboxyl group as a center for interaction with the antibody. A hapten also requires, in precise spatial relation to the carboxyl group, a carbonyl group presumed to serve as a hydrogen acceptor for hydrogen bonding to the antibody. The activity of *cis* but not *trans* compounds analogous to succinylate clearly shows the structural requirement; succinylate, itself, and homologous haptens are believed to be held in the *cis* form by internal hydrogen bonding of the imino and carboxyl groups. The contribution of van der Waal's forces to the hapten-antibody interaction is shown by the thirtyfold decrease in the inhibition constant resulting from replacement of the phenyl group of the hapten by a methyl, and the further decrease on replacing the methyl by hydrogen. The increasing inhibitory effect of para substituents on the benzene ring in the order $H < NH_2 < Br < N:NC_6H_4OH < NO_2$ is also in accord with usual van der Waal's effects. Inhibition of interaction by solvation is held responsible for the nonreactivity of the more polar tartranyl derivative.

This significant development of our understanding of specificity shows that biological reactions may depend upon rigid spatial relations between interacting groups. An illuminating nonbiological parallel is the isomerization of butene-1 to butene-2 (134). This reaction is catalyzed by only those acids meeting (like the haptens) rigid structural requirements. The acid must have two functional groups, one able to accept a hydrogen atom, the other able to release another hydrogen atom at a distance of very nearly 3.5 Å away. Our understanding of specific reactions therefore agrees with Ehrlich's simple lock and key model and also with Langmuir's contention that the spacing of atoms on the surface of a catalyst is the critical factor for adsorption and reaction.

Short range as opposed to long range forces.—Extensive study of protein interactions shows that most of them at least follow known laws of short range interactions of other molecules, the equilibrium reached being determined by the balance of attractive and repulsive forces regulated by the component factors of group interaction

such as dispersion, induction, polarization, steric hindrance, and solvation. Though interaction may be favored by energy considerations, the groups may be prevented from approaching to the necessary distance by steric hindrance, solvation, chemical blocking, or incorporation in a crystal structure.

The possibility, suggested by Rothen (135), that biologically specific reactions can occur at distances up to 200 Å through barriers of inert material seems improbable from experiments of Karush & Siegel (136), who find that electron micrographs of colloidion replicas of a film of six superposed monolayers of serum albumin show great irregularity, with pits and ridges commonly varying in height by 50 to 85 Å and in extreme cases to more than 1,000 Å. Films of such irregularity may well permit usual short range interaction at what might appear to be long range separation.

Nevertheless, long range nonspecific interaction does occur in some protein systems, for example in solutions of tobacco mosaic virus (137), where the molecules may assume regular positions at intervals as great as 1,000 Å. In hemoglobin crystals (47) molecular layers may be separated by water to distances of 65 Å. In these instances transmission of the forces of interaction through the intervening water may reflect the interaction of protein and water. Thirty per cent of the water of hemoglobin crystals is so firmly bound that it is not available as a solvent for small ions (138).

Protein purity and protein interaction.—The high affinity of proteins for various molecules is a matter of concern for protein purity. It is apparent that an impurity can be bound to a protein without preventing its crystallization, and with such stability that the complex can appear to be an equally well-defined species by thermodynamic, kinetic, and even biological criteria. Such contamination may easily escape analysis unless specifically sought, but may nevertheless mask some chemical or biological properties of the parent protein or produce other properties not characteristic of it. How many of the conjugated proteins result from interactions that have occurred during their removal from their natural environment? A suggestive instance, discussed elsewhere (p. 111) in this volume, is the binding of lipid by wheat gluten (139), which is shown by nonextractability by ether to occur when the moisture content of the flour rises above a critical value near 30 per cent.

Interaction with water.—The interaction of proteins with water, the mechanism of which is still incompletely understood, is a matter

of both theoretical and practical importance. Many recent papers deal with the interpretation of the familiar sigmoid curve of water content as a function of vapor pressure. During the past year Robinson (140), Benson & Ellis (141), and Davis & McLaren (142) have found the Brunauer-Emmett-Teller treatment to give a fair account of the water adsorption of several proteins over a restricted pressure range. Davis & McLaren find marked differences, especially between soluble and insoluble proteins, in the partial molal free energy, net heat, and net entropy changes relating to the sorption of water. Most proteins show a negative net entropy at low vapor pressures [see also Cassie (143)], presumably due to orderly packing of the water on the protein. The positive entropy effect shown by lyophilized β -lactoglobulin and egg albumin is attributed to incipient solution of the protein.

Detailed interpretation of the water adsorption of proteins may be attempted following the suggestion of Simha & Rowen (144) that the classical theory of adsorption on specific sites be applied at low vapor pressure and the statistical theory of mixing of high polymers with solvents at the other extreme, the intermediate region being handled as an even transition between these conditions. Barrer (145) has already shown that combination of adsorption and solution theories can lead to a sigmoid isotherm.

Because the high affinity of proteins for water and the usual large negative entropies of interaction are conditions not considered in the derivation of present treatments of high polymer-solvent interaction, little is gained by applying these theories in their present form to protein-water systems (146). The Flory-Huggins treatment at best succeeds only for the more ideal systems such as rubber-benzene.

Proteins are complicated by the varying accessibilities of adsorbing sites as well as their various kinds. The result is like the capillary pore effects found with other adsorbents in fibrous form. We can expect that a better account of water adsorption can be given as more precise knowledge is available of the nature and location of these sites. The effect of molecular size and crystallinity on the binding of water by peptides has been described (120). Mellon *et al.* (147) also estimate that about half of the water adsorbed by casein from the vapor phase is bound by the peptide linkage and about one-fourth by amino groups.

Wright (148), in measurements of the low angle x-ray diffrac-

tion of collagen, finds the fundamental spacing of 628 Å at 2 per cent relative humidity increased to 672 Å at saturation.

INTERCHAIN FORCES AND PROTEIN STABILITY

The limitation by crystallinity of the interaction of fibrous proteins with water has been cited. When the chains are relatively regular and fit together well, as in silk, the high crystallinity leads to reduced water binding and high wet strength. But when, as in most proteins, the extended chains have bulky groups attached which hinder crystallinity, water penetration and adsorption are high and the fibers weak unless the chains are otherwise stabilized, as in wool, by chemical crosslinks (149). Therefore, two prime objectives of technological protein research are the study of cross-linking agents and methods, to improve the utility of protein fibers and plastics, and the development of mild processing conditions that will degrade the proteins as little as possible.

Crosslinking with formaldehyde.—Fraenkel-Conrat & Olcott, by liberal use of model substances, including proteins, clearly show the probable nature and conditions of formation, by formaldehyde, of covalent methylene links between amino groups and, on the other hand, amide, guanidine (150), phenol, imidazole, and indole (151). Selective acetylation of amino groups prevents the crosslinking, which otherwise occurs more readily with amide and guanidine groups than with the cyclic groups. An interesting application (150) is the use of ammonia or primary, but not secondary, amines with formaldehyde as difunctional crosslinking agents for proteins, like zein and gliadin, rich in amide but relatively poor in amino groups. By osmotic pressure measurement Fraenkel-Conrat & Mecham (152) show the increased molecular weights of formaldehyde-treated proteins to be dependent upon the presence of free amino groups in the original protein. Such studies not only show the possible mechanisms by which formaldehyde hardens or tans proteins, but demonstrate ways of combining low molecular weight amines, amides, phenols, etc., with proteins to make a wide variety of stable derivatives.

Swain *et al.* (313) have reported upon the kinetics of formaldehyde binding by casein. Maximum binding, depending on pH and temperature is about two molecules per lysine residue, or somewhat less than one per basic or amide group. Some was not reversibly bound, implying formation of some carbon-carbon bonds.

Solubility, unfolding, and interchain forces.—The opposing forces of chain interaction and thermal agitation and solvation determine protein solubility. Feather keratin dissolves (153) above a critical temperature in a mixture of alcohol, water, salt, and reducing agent appropriate for the dissociation of the several types of interchain forces which stabilize the natural material. Silk dissolves in cupriethylene diamine through cleavage of the stabilizing hydrogen bonds (154).

Native proteins in general dissolve as corpuscular units provided the degree of interaction with the solvent is not too great; otherwise unfolding occurs (155). Though we do not know the details of the specific folding of native proteins, our present idea of their structure seems correct. Corpuscular and fibrous proteins are stabilized by similar forces which provide an energy barrier of magnitude greater than the forces of thermal agitation. A given protein structure is stable only so long as the solvent does not destroy this barrier and the temperature remains low enough that the kinetic energy is small compared with the barrier energy. When this barrier is overcome, thermal forces release the chains into forms consistent with the mechanical stress, degree of solvent interaction, and bulk and charge distribution of the chains; completely unrestrained flexible chains would assume random forms.

Boyd & Eberl (319) have applied absolute reaction rate theory to the kinetics of heat denaturation of tobacco mosaic virus. From computed activation energies they infer that the equivalent of 25 to 30 hydrogen bonds must be broken to start denaturation, probably through a chain mechanism.

Pliability and contractility of proteins chains is expressed in the long range elasticity shown by fibrous proteins, especially when chain interaction is reduced by solvent swelling (156). Energy to extend coiled, pliable chains can be supplied (149): (a) externally by tensile stress; (b) internally, by linear interaction, crystallization, of neighboring chains; (c) by electrostatic repulsion of similarly charged groups (157, 158); or (d) by interaction of the chains with a "good" solvent (159). The recognized uncoiling of protein chains in urea is an example of the last.

Whewell & Woods (160) cite an effect attributed to ion interaction. A wool fiber in cuprammonium solution binds copper, as shown by the color, and contracts to about 30 per cent of its initial

length. The color and contraction remain on prolonged washing with water, but in dilute acid the color disappears and the initial length is restored. The contraction appears due to the binding of interacting centers along the chain by copper.

Mechanics of muscle behavior.—Reversible extension of protein chains is a plausible basis for muscle contraction. Meyer (161) has proposed the electrostatic repulsion of ionized acid or base groups to account for extension. This mechanism is hard to reconcile with the small physiological pH changes. Kirkwood & Riseman (162) have adapted the idea of electrical repulsion to the use of energy of carbohydrate metabolism through the transfer of phosphate. Phosphorylation of the hydroxyl groups of myosin or actomyosin would provide a series of highly charged phosphate groups that would favor extension of the chains by mutual repulsion. Contraction would occur spontaneously on dephosphorylation. The correspondence of the observed change in the elastic modulus of muscle to that estimated from electrostatic considerations, assuming phosphorylation at sites 100 Å apart (in accord with the serine content of myosin), supports this mechanism.

Although this mechanism agrees with the known behavior of linear flexible chains, the complexity of muscle requires an account of the interplay of the other proteins and ions involved (163). A more serious criticism is that configurational changes of protein chains have relatively long relaxation times compared with some rates of muscular response. Therefore Kirkwood & Riseman (162) and Bull (164) suggest an alternative, based on linear aggregation and dissociation of corpuscular units.

Linear association.—Instances of linear association are encountered in protein systems, including actomyosin. Feuer *et al.* (165) emphasize the striking ability of corpuscular actin to aggregate to a linear form, either on addition of inorganic ions or on change of pH. Polymerized actin gives highly viscous solutions with strong flow birefringence; with myosin it forms actomyosin. Threads of actomyosin, like muscle fibers, contract in the presence of adenosinetriphosphate and inorganic ions.

Polymerization of actin is particularly sensitive to magnesium ions (165, 167). Oxidants prevent aggregation; if oxidation is not too drastic the effect is reversible. It is suggested that magnesium ions combine with the oxidizable groups to join together the corpuscular units. Although the nature of this oxidizable group is not

known, Bailey & Perry (166) have shown that thiol groups in myosin are required for interaction of actin and myosin; when these are oxidized, interaction is prevented and triphosphatase activity as well. From the effects of adenosinetriphosphate, magnesium, and calcium on the viscosity of myosin, Mommaerts (167) infers that the effect of adenosinetriphosphate does not depend on enzymatic hydrolysis. Munch-Petersen (320) finds adenosinetriphosphate at 0.05 mg. per ml., for a given applied force, to double the area of myosin monolayers. The reaction is specific, for other phosphates require 10 times this concentration for a similar effect; actomyosin is less active than myosin and actin is inert.

From light scattering data supported by electron micrography, Jordan & Oster (168) show actomyosin to have a configuration intermediate between those of a stiff rod and random coil. Adenosinetriphosphate changes the shape to a more randomly coiled form. Therefore, mechanisms specifying dissociation of the protein by adenosinetriphosphate are questioned, but it is still possible that dissociation of the actin component of the complex might occur without its being separated from the myosin.

Binkley (169) has studied the interaction of crystalline myosin and purified fibrous actin by the changing flow birefringence of their solutions. The normal combining ratio was near three parts to one on the basis of nitrogen content. Adenosinetriphosphate caused marked decrease in the flow birefringence of either myosin or actomyosin.

Waugh (170) finds the fibrils formed by acid treatment of insulin to be due to linear aggregation to lengths of tens of thousands of Ångstrom units, the widths averaging 150 Å. These fibrils can further interact to form spherules characteristic of heat precipitated insulin, or, with dilute alkali, may revert to a crystallizable product which is like normal insulin in molecular weight, biological activity, and in all other properties tested.

By treating tobacco mosaic virus solutions with serum albumin of similar charge, Lauffer (171) has produced mesomorphic fibers composed almost entirely of virus and similar to those sometimes observed in the stored juice of diseased plants.

From low angle x-ray diffraction, Kratky (172) finds evidence of linear association of *Helix pomatia* hemocyanin. It is apparent that linear aggregation of corpuscular proteins may be a general phenomenon of real importance in biological systems. Winter

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(173) proposes a theoretical basis.

Association and the molecular size of proteins.—Molecules of corpuscular proteins often appear to consist of several more or less firmly joined peptide chains. Sometimes these chains may be dissociated by dilution, by being taken beyond the pH region of stability, or by solution in such reagents as urea. In other instances the chains may be prevented from dissociating by covalent cross-links. By end group analysis Hoover *et al.* (174) found bovine serum albumin to have four chains of average molecular weight 18,000; β -lactoglobulin, four averaging 8,400; and casein, three or four averaging 9,000 to 14,000, depending upon the preparation. The zein molecule appears to have a single chain of about 23,000. Dimerization of tetanus (175) and botulinum type A (58) toxins appears to occur. The synthetic polypeptides of apparent very high molecular weight, prepared by a modified Leuchs' polymerization (176), were found by Eirich & Katchalski (177, 178) to dissociate to units of average molecular weight 10,000 in appropriate solvents.

Synthetic polypeptides and protein structure.—Katchalski *et al.* (179) report the synthesis and properties of polylysine and Hanby *et al.* (222), polyglutamic acid. Astbury *et al.* (180) have given a preliminary account of a very significant comparison, by x-ray, infrared, and associated methods, of synthetic polypeptides and copolymers to proteins of related composition. By the Leuchs' polycondensation they have made many polypeptides from glycine, sarcosine, various optically resolved or racemic amino acids, and their mixtures. X-ray diagrams of most preparations could be interpreted as modifications of the β -keratin pattern, with small variations in the backbone spacing as well as expected variations due to side chain differences. Some polymers, especially of optically active monomers, showed larger backbone spacings, tentatively attributed to presence of water molecules between chains. In all instances, infrared absorption showed predominantly the presence of the same degree of interaction between CO and NH of neighboring chains as in proteins, so that the corresponding distances must be closely similar. Preparation of oriented films succeeded especially in the case of the copolymer of D-leucine and DL-phenylalanine. Confirming and extending work of Brown, Coleman & Farthing (181), patterns resembling alpha diagrams of unstretched keratin were observed, but these could not be converted to a beta form. Infrared absorption bands at certain frequencies characteristic of

the amino acids persist in polymers and copolymers, and therefore probably in proteins. In this way glycine, alanine, and probably tyrosine have been identified in intact silk fibroin.

PROTEIN DEGRADATION STUDIES

Analyses of proteins.—By analyses of amino acids containing refractory nitrogen and of proteins rich in these, White *et al.* (287) show the suitability for protein materials of a proposed micro-Kjeldahl method using mercuric oxide catalyst and 80 min. digestion. Frey (184) has studied a semimicro method and compared results from copper and selenium catalysts with theoretical values and those found by the Dumas method.

The ninhydrin reaction has been studied by Schönberg *et al.* (185). Moubasher (186) suggests analogous use of the regenerable and less expensive perinaphthindan-2,3,4-trione hydrate.

Reactions of proteins depend finally on their detailed structure, so that understanding of these reactions often requires exact knowledge of the amino acid residues present and the ways in which these are joined. As analyses become precise, analysis becomes a reliable test of identity and permits differentiation of nearly related proteins. Knight (182) found an apparent stepwise variation of a few amino acids among different strains of tobacco mosaic virus. Csonka (183) may have another example in hen's egg albumin prepared from the eggs of hens fed different diets.

Protein analyses are usually analyses of hydrolysates, and unsatisfactory to the extent that components may be changed during hydrolysis; also their order of connection is obscured. The first defect is illustrated by a study of Kuiken *et al.* (315) of the oxidative destruction of tryptophane. Similarly, Olcott & Fraenkel-Conrat (316) show explicitly that the cysteine content of hydrolysates need not correspond directly to that of the protein, especially because of cleavage of cystine by tryptophane under usual acid hydrolysis conditions.

Amino acid analysis by colorimetric or isolation methods.—Developing known methods, Michel *et al.* (187) applied ninhydrin to microanalysis for glycine in protein hydrolysates through colorimetric estimation of the liberated formaldehyde. Alanine has been similarly measured (188, 189) and through conversion to lactic acid (190). Michel & Bozzi-Tichadou (191) measured small amounts of serine in protein hydrolysates by the formaldehyde produced by periodic acid.

Nakamura & Binkley (192) have reported a new colorimetric method for cysteine, using brucine and potassium persulfate. The method is more specific than that of Sullivan & Hess in giving no test with cysteinylglycine. The only reported interfering substances are ascorbic acid, epinephrine, thiourea, and heavy metals. Diazo-tized N¹-diethylsulfanilamide is suggested (193) as a reagent for thyroxine.

Heathcote observed a coprecipitation of lysine flavianates (194) with the arginine and histidine salts from protein hydrolysates and confirmed the isolation of hydroxylysine (195) as the picrate from gelatin hydrolysates. Though lysine and hydroxylysine picrates form solid solutions, they were distinguished by chemical analysis, and the free acids by periodic acid oxidation and by the selective action of L-lysine decarboxylase.

By colorimetric and isolation methods, Slack (196) estimated 10 amino acids of whole potato and of an electrophoretically impure globulin, tuberin. This sort of analysis can often be done conveniently by microbiological methods or more comprehensively, especially for routine use with small samples, by chromatographic methods.

Microbiological amino acid analysis.—Virtanen & Louhivuori (197) have studied the use of aspartase-containing extracts of *Pseudomonas fluorescens* to estimate aspartic acid. Typical microbiological methods estimate the quantities of individual amino acids, supplied in limited amounts, by their effects upon the growth of suitable organisms. Specific studies of the metabolism of amino acids by bacteria, such as that of Riesen *et al.* (198) with cystine, have led to assay methods for valine (314), phenylalanine (303), leucine (199), and histidine (304), as well as more general methods (305, 306). Meinke & Holland (307) report instances in which threonine and serine affect each other's metabolism. Other examples of antagonism (308) exist. Amino acid derivatives have been explored as competitive bacteriostatic agents (309).

Using microbiological methods, Smith & Greene (200) have checked the isoleucine contents of several proteins to correct findings derived from a standard containing the *allo* isomer. They report less isoleucine in β -lactoglobulin than Moore & Stein (7), and much less than Stokes (201) or Brand (82) and their associates. Solomon *et al.* (203) have analyzed cerebrospinal fluid for 11 free amino acids, using extended heating, after removal of proteins, to destroy antibiotic activity. Thompson & Kirby (310) met a similar

problem by finding standard growth curves in the presence of toxic materials. Sarkar *et al.* (204) have published analyses of bovine sperm and seminal plasma.

Studies by Woolley (312) and Krehl & Fruton (311) of bacterial utilization of various peptides begin a novel synthetic approach to the structure of the growth stimulating streptogenin grouping occurring in various proteins.

Amino acid analysis by adsorption and partition methods.—An extensive trend in present amino acid analysis is the development of chromatographic techniques for resolution of mixtures. Williams (205) gives an extensive review of adsorption or partition analysis, in columns and on paper. The New York Academy of Sciences (206) has published a series of discussions of various aspects not restricted to protein chemistry. A symposium of the Biochemical Society has appeared in summary (202).

Adsorption analysis in columns.—Column chromatography is the traditional method. Glueckauf & Coates (207 to 210) have developed the theory of adsorption separations of systems having isotherms of various characters. Weiss (211) describes the modification of adsorbents by pretreatment with strongly adsorbed substances to improve recovery of related substances. Claesson has developed Tiselius' analysis of the effluent liquid, both theoretically (212) and instrumentally (213). Using frontal analysis, Syngé & Tiselius (214) adduced evidence that the polypeptide gramicidin is a single substance, although gramicidin S and tyrocidin are complex, and tyrothrycin a mixture of tyrocidin and gramicidin. Differences in composition were shown by paper chromatography (discussed later) of the respective hydrolysates. Drake (215) records automatically the separation of glutamic and aspartic acids. Jutisz & Lederer (216, 217) described adsorbent-solvent systems for making group separations of peptides and amino acids, the groups to be analyzed separately by paper chromatography. Riley (218) has even used chromatography on silica to isolate active Rous sarcoma virus. It is of special interest that these methods permit practical analytical differentiation of peptides.

Ion exchange media may be specially suited for quantity separations even when the substances to be separated are very similar. The composition of the effluent liquid can be deduced from theoretical treatment like that used for fractional distillation or extraction. Mayer & Tompkins (219) have developed the theory with particular reference to separation of the rare earth elements.

Appelzweig (220) discusses the general laboratory use of ion exchange agents and Bendall *et al.* (221) the separation of various organic bases and amino acids with cation-exchange resins.

Partition chromatography.—When the adsorbency of the supporting material is in abeyance, a separation may be made with a solvent of limited miscibility with water, with results suggestive of a continuous partition equilibrium between the washing liquid and the water held by the supporting phase. The effect is that of a large number of liquid-liquid partitions on the counter-current principle, so widely explored by Craig (5) and his associates. Counter-current partition has been applied by Gregory & Craig (6) to the separation of a gramicidin preparation into four components, of which three were then crystallized. Keston *et al.* (223) have used this method to separate and estimate glycine, alanine, and proline, as radioactive derivatives, from protein samples as small as 1 mg.

Although chromatography on starch, silica, or paper may closely approach the behavior of a partition system, as noted by Martin (224), in other instances Moore & Stein (7) note prominent adsorption effects resulting in the separation of amino acids of a mixture passed through a column in an order different from that of their partition coefficients. In making such separations, Moore & Stein found it convenient to use an automatic fraction collector (7, 8). Darmon *et al.* (225) estimated the proportion of acetylated leucine and isoleucine in a fraction, separated chromatographically, from differences in their infrared adsorption.

Paper chromatography.—Filter paper in sheet form is widely applied as a chromatographic supporting medium. Martin (226) has reviewed the theory and methods, and Consden (227) the scope and results of its recent application. Longenecker (228) and Winsten (229) described special apparatus. Capillary rise may be used instead of gravity to move the liquid over the support, as described by Horne & Pollard (230) and by Williams & Kirby (231), who have an attractively simple method using no specially made apparatus. Rutter (232) describes development of a mixture in concentric rings on filter paper.

Consden, Gordon & Martin (233) have described an apparatus for removing inorganic salts from protein hydrolysates by electrolysis. Smith & Page (234) have developed the use of long chain amines in chloroform solution to remove acids from solution, a method also applicable to hydrolysates. Desirable preliminary

fractionation of amino acid mixtures can be made by column chromatography with ion-exchange resins, the method being given by Appelzweig (220), Consden *et al.* (235, 236) and Hems *et al.* (237). Butler & Stephen (238) and Consden *et al.* (233, 239, 240) have used electrophoresis of amino acids for making preliminary separations. The developing solvent is often specially chosen, examples being cited by Consden *et al.* (233), Dent (241), and Williams & Kirby (231), but phenol, followed by collidine moving in the perpendicular direction, is usually used. Interfering color, attributed to copper, is said by Williams & Kirby (231) to be avoided by purifying the solvent. Haugaard & Kroner (242) have applied an electric current at right angles to the direction of solvent development to provide additional resolution of amino acids according to their acid, basic, or neutral character.

The resolved amino acid spots are conveniently revealed by ninhydrin, use of which is described by Dent (241), and Pratt & Auclair (243) among others. Consden *et al.* (233) apply ninhydrin to one of replicate analyses to locate spots on the others. Woiod (244, 245) states that it may be applied lightly enough not to interfere seriously with later analysis. Phillips (246) shows that ultraviolet light may be used to find the amino acid spots, although ninhydrin is more sensitive. Dent (241) reports diazotised sulfanilic acid to be a better reagent for histidine and tyrosine. Under appropriate conditions, radioactive substances can be found, identified, or estimated quantitatively either by a device for scanning the paper with a counter [Fink & Fink (247), Keston *et al.* (248), and Tomarelli & Florey (249)], by testing consecutive strips [Keston *et al.* (250)], or by radioautography [Fink *et al.* (247, 251) and Keston *et al.* (248)].

The chemical nature of the resolved substances may be shown in part by the ninhydrin reaction. They may often be provisionally identified by the ratios of their movements to that of the solvent. Tables of these R_F values are given by Consden *et al.* (233), Pratt & Auclair (243), and Williams & Kirby (231), among others. Dent (241) and his associates (264) have published maps for two-dimensional analyses. Jones (252) has used the D-amino oxidase from sheep kidney to demonstrate the D or L configuration of amino acids. Winsten & Eigen (253) have used microbial growth to indicate the position or nature of growth factors; this method might be extended to biologically active amino acids or peptides, whether stimulating or inhibitory.

To confirm the identity of any resolved component, comparative analyses are carried out with known substances, either added directly to the mixture, as by Consden *et al.* (233), or in parallel experiments. Components have also been extracted from their spots and their identity confirmed, by electron diffraction [Polson *et al.* (254)], and by x-ray diffraction [Christ *et al.* (255)]. Identification of peptides from partial hydrolysates has been accomplished by Consden and his associates (224, 233, 240, 256) by hydrolysing material from a peptide spot, with and without destroying the free amino group chemically, and making a new chromatogram of the hydrolysate.

Fisher *et al.* (257) and Polson *et al.* (254) have discussed the estimation of amounts of separated components from the dimensions of the spots, as may be done conveniently by comparison with spots containing known quantities and in serial dilution experiments. Fisher *et al.* also show it to be possible to take microphotometer readings of a photographic print of the developed, ninhydrin-treated spot along a line through the darkest portion, and use the area under the density *versus* distance curve as a measure of concentration. Polson *et al.* do not recommend micro-Kjeldahl analysis of material leached from a spot because of possible contaminants from the paper; however, these authors and also Naftalin (258) describe photometric measurement of the ninhydrin reaction products. Alpha amino acids may be extracted as copper complex salts by treatment with copper phosphate as described by Woiwod (244, 245). Other amino compounds, not extracted, may then be demonstrated with ninhydrin, according to Dent [cited by Consden (227)]. The copper complex may be estimated colorimetrically, or by titration [Consden *et al.* (235)], or polarographically [Jones (259), Martin (224, 260)].

Keston *et al.* (248, 250) estimate amino acids by the radioactivity of resolved *p*-iodobenzenesulfonyl derivatives containing radioactive iodine or sulfur. Identity and purity are confirmed by eluting individual spots, adding a known inert carrier, and showing maintenance of activity during purification. Even if complete resolution is not attained, the concentration can be measured by applying the isotope dilution principle.

Paper partition chromatography is simple enough to be used routinely, especially in studies where quantitative analysis is not needed: for example, to test the purity of synthetic peptides [Synge (261)]; to control the separation of amino acid mixtures

on a larger scale by ion-exchange columns [Hems *et al.* (237)]; or for the control analysis of nutrient media in antibiotic production [cited by Consden (227)]. The small samples required make it ideal for surveys of amino acids, free or combined, in plant tissues [Allsopp (262); Auclair & Jamieson (263); Dent *et al.* (264); La-Cour & Drew (265); and Lugg & Weller (266), who have also used older methods (267)]. Fink & Fink (247) have identified several radioactive amino acids formed in *Chlorella* by photosynthesis in the presence of radioactive carbon dioxide. Studies reported by Calvin (268) show these to be secondary products.

Hydrolysates of trypsin and of the trypsin inhibitor from soybeans have been studied by Work (269) and sperm proteins have been analysed by Hultin (270). The paper partition method is specially suited for quick discovery of striking features of composition such as deficiencies or absences of particular amino acids or the presence of unusual components. Polson (271), for example, has tentatively identified two of three unfamiliar components of *Escherichia coli* hydrolysates as α -amino-*n*-butyric acid and an isomer of α -aminoheptylic acid. Catch & Jones (272) and Jones (252, 273) have shown aerosporin hydrolysates to contain 2,4-diaminobutyric acid, D-leucine, and L-threonine.² Fink & Fink (274) have provisionally identified 3-iodotyrosine as a principal thyroid substance formed on injection of radioactive iodide. Lewis & Alderton (275) find subtilin hydrolysates to contain lanthionine as well as unidentified sulfur compounds. Lindley (276) also finds unaccounted-for sulfur in certain keratins.

Details of several metabolic processes have been established. Dent & Schilling (277) report evidence that several proteins, but not homologous plasma protein, are absorbed by dogs into the blood stream mainly or entirely as the simple amino acids. Winsten & Eigen (253) confirm the amination of pyridoxal by amino acids, with the exception of cysteine, which was shown to react differently. Borsook *et al.* (279) demonstrated a transformation of lysine to aminoadipic acid by guinea pig liver. The chromatography of urine has been explored for clinical meaning by Young & Hamburger (280) and has indicated the origin of aminobutyric acid in methionine [Dent (241, 281)], and the metabolism of methionine in the detoxification of benzene and bromobenzene [Tomarelli & Florey (249)].

² D-threonine according to the revision in nomenclature proposed by the Committee on Nomenclature of the National Research Council.

The most significant application of chromatography chemically has been to polypeptides and partial hydrolysates, giving a workable, systematic method of studying the order of amino acid residues, as by Consden *et al.* (233). Using methods mentioned, Consden *et al.* (256) have proven that gramicidin S contains the repeating pentapeptide unit, α -(L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-propyl, by identification of four of the five possible dipeptides and some tripeptides consistent with the given structure. In the same way tyrocidine (282) is shown to have the unit, valyl-ornithyl-leucyl and probably aspartyl-glutamyl-tyrosyl. Wool yields a variety of acid peptides (240, 283) from which it appears that a substantial quantity of the glutamic acid present is grouped in regions with two or more glutamic acid units joined together.

Kinetics of hydrolysis.—From the kinetics of acid hydrolysis of egg albumin, Bull & Hahn (284) infer that 56 of the 400 peptide bonds of this protein are more susceptible to cleavage than the rest. Desnuelle & Casal (118) report serine and threonine peptide bonds to be especially readily hydrolysed by acid. Therefore, it is interesting that Rees (285) finds 8.14 per cent serine and 3.83 per cent threonine in egg albumin, corresponding to 49 residues per molecule of weight 45,000, suggesting approximate agreement of the analytical and kinetic studies.

By a new surface-film pressure method, Bull & Hahn (286) have estimated the molecular weight distribution of partial hydrolysates. Although the size distribution of peptides from acid hydrolysed egg albumin differed from that expected from random hydrolysis, no accumulation of peptides of a given weight was noted. Brief hydrolysis gave little material of molecular weight in the range 10,000 to 30,000, suggesting that the initial attack may be at or near the end of the peptide chains. Another alternative is that once attack is begun, a molecule is rapidly degraded to units of molecular weight less than 10,000.

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NUCLEOPROTEINS, NUCLEIC ACIDS, AND DERIVED SUBSTANCES¹

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Despite the enormous output of published work during the last few years, it is only too clear that our knowledge of the nucleic acids is still at a very elementary level. The structure of no nucleic acid is as yet properly established; we do not even know how to prepare a homogeneous specimen containing one molecular species. Nor do we know whether all nucleic acids of the pentose type (or of the desoxypentose type) prepared from different sources are identical or not; it would even be difficult to decide what criterion to apply at the present time. And, although some evidence is now forthcoming, we are still woefully ignorant of the biological function of the nucleic acids. Nevertheless, the information at our disposal is steadily increasing and much published work has appeared since this subject was reviewed in the last *Annual Review of Biochemistry*. The most important publication has undoubtedly been the report on the Cold Spring Harbor Symposium on Nucleic Acids & Nucleoproteins (1) which was held in 1947. This volume contains a wealth of information in its 279 pages and reference to many of the papers in it will be made in this review. Shorter reviews of the nucleic acid field have also appeared (2, 3).

NUCLEIC ACID STRUCTURE

The question of nucleic acid structure has been admirably reviewed by Gulland (4, 5). He has pointed out that while the bases adenine, guanine, cytosine, uracil, and thymine are known to occur in nucleic acids, no systematic search for other breakdown products has been undertaken and that the presence of other breakdown products cannot be excluded. One such product may be the "epi-cytosine" found by Hotchkiss (6) by paper chromatography of the hydrolysis products of thymus desoxypentosenucleic acid.

It is necessary to repeat the warning given last year (7) against the indiscriminate use of the terms ribonucleic acid and desoxy-

¹ This review covers the period from November, 1947 to October, 1948.

ribonucleic acid. The assumption continues to be made that all pentosenucleic acids are identical with yeast ribonucleic acid and it may well be that future work will prove this assumption to be correct, but at the present time the justification is slender. The pentose sugar has been established as D(-)-ribose in yeast pentosenucleic acid by identification of the corresponding aldonic acid as ribobenziminazole (8) and in liver pentosenucleic acid by identification as the *p*-bromophenylhydrazone (9). Guanylic acid, uridylic acid, and cytidylic acid have now been isolated from tobacco mosaic virus nucleic acid and shown to be identical with their analogues from yeast pentosenucleic acid (10). In the case of these three nucleic acids, therefore, the use of the term "ribonucleic acid" is justified but until further evidence is available it would be well to use the general term "pentosenucleic acid" (PNA) in preference to "ribonucleic acid." In the same way it is desirable to use the general term desoxypentosenucleic acid (DNA) in preference to desoxyribonucleic acid since the sugar has been proved to be desoxyribose only in the case of calf thymus material. These points have already been stressed by Chargaff (7, 11).

It is also tacitly assumed that uracil is present in all pentosenucleic acids, although its occurrence has until recently been established only in the case of yeast PNA. Uracil has now been isolated from liver PNA (12) and its presence in tobacco mosaic virus PNA has also been proved (10).

Although individual nucleotides from yeast PNA and tobacco mosaic virus PNA are identical, it does not follow that the nucleic acids themselves are identical. Indeed they differ markedly in particle size, and although all nucleic acid preparations are probably inhomogeneous, Schwerdt & Loring (10) regard it as improbable that the two nucleic acids are identical in their respective nucleoproteins. Gulland (5) has pointed out that two polynucleotides would be chemically and perhaps biologically distinct, although they contained the same nucleotides in the same proportions, if the relative positions of even two nucleotides in the polynucleotide chain of one of them were interchanged. Moreover, the evidence from specific nucleoproteins (4, 5, 13 to 18) suggests that both nucleic acid and protein may contribute to the specificity. Loring (19) even suggests that the composition of a particular PNA may be characteristic less of the organism or tissue of origin than of the stage of growth involved.

Further evidence discrediting the statistical tetranucleotide hypothesis has come to light. The term "statistical tetranucleotide" was proposed by Gulland, Barker & Jordan in 1945 to designate the presence of four nucleotides in equimolecular proportions. Gulland, Jordan & Threlfall (5, 20) have found that in yeast PNA the average ratio purine nitrogen/pyrimidine nitrogen was 1.86 instead of 2.0 as required for a statistical tetranucleotide. This might indicate the presence of more pyrimidine than purine nucleotides or, more improbably, that some of the uridylic acid is replaced by cytidylic acid. For thymus DNA the ratio purine nitrogen/pyrimidine nitrogen was 1.60 and electrometric titration data indicated that for every four atoms of phosphorus there was one guanine radicle, one of thymine, 1.2 of cytosine, and 0.8 of adenine. Excess of guanine over adenine has also been recorded by Loring and his colleagues (21), who have in addition found amounts of pyrimidines less than that required by the tetranucleotide hypothesis (19).

Scott (22) has published electromicrographs of sodium desoxypentosenucleate. They indicate the occurrence of lateral and longitudinal aggregations of the nucleic acid columns.

Action of ribonuclease.—Loring *et al.* (19, 21) submitted yeast PNA to the action of ribonuclease under conditions in which the liberated nucleotides were removed by dialysis leaving a non-dialyzable fraction resistant to further ribonuclease action. This resistant fraction was separated into two parts, one precipitable by 85 per cent acetic acid and the other precipitable from the filtrate from the first by addition of alcohol. These ribonuclease resistant fractions in comparison with the original PNA showed an increase in the relative amount of guanine present and a low pyrimidine content indicating that the enzyme is concerned with the liberation of pyrimidine nucleotides rather than purine nucleotides. This conclusion is supported by Schmidt, Cubiles & Thannhauser (23, 24, 25), who isolated from the products of the ribonuclease digestion of yeast PNA a polynucleotide fraction with a purine nucleotide/pyrimidine nucleotide ratio of 2:1. When this fraction was treated with prostate phosphatase which acts exclusively on pyrimidine nucleotide groups, a polynucleotide fraction was obtained containing exclusively purine nucleotides. It is tempting to suppose that pancreas PNA reported by Jorpes (26, 27) to contain an unusually high purine content, is in some way related to these

ribonuclease resistant fractions since during extraction of the PNA the possible action of the enzyme, which is exceedingly abundant in pancreas, cannot be excluded.

The enzymatic degradation of nucleic acids has also been discussed by Greenstein *et al.* (28 to 31) and by Zittle (32 to 35).

The activity of ribonuclease towards the pentosenucleoproteins of yeast is inhibited by penicillin (36). Acridines and streptomycin also inhibit ribonuclease by forming electroadsorption complexes with the pentosenucleoproteins (37, 38).

THE NUCLEIC ACID CONTENT OF TISSUES

Stumpf (39) has described a specific colorimetric method for the determination of DNA based on the development of a pink colour with cysteine and sulphuric acid. PNA does not interfere.

In the usual orcinol method for the estimation of pentose in PNA the sugar moieties of the purine nucleotides alone react. By treatment with bromine at 105°C. Massart & Hoste (40) find that the pyrimidine-bound pentose can also be made to react with orcinol.

A method for the determination of purine nitrogen in quantities of the order of 10 to 40 $\mu\text{g.}$ and its application to nucleic acid analysis is described by Vendrely (41), while Di Carlo & Schultz (42) have discussed the quantitative determination of yeast PNA by spectrophotometry.

A method for the determination of nucleic acids in cytological preparations is described by Pollister & Ris (43). It is based on the photometric measurement of absorption at 2537 Å by both types of nucleic acid followed by removal of the nucleic acids by treatment with hot trichloroacetic acid according to the principle of Schneider (44) so that the same preparation may then be used as a blank. The ratio between the two types of nucleic acid is determined by staining with pyronine-methyl green mixture, while protein may be determined in the cytological preparation by the quantitative use of the Millon reaction. Using this technique Ris (45) has measured the composition of chromosomes during mitosis and meiosis, while Pollister & Ris (43) have found that one thymus nucleus contains 1.1×10^{-9} mg. nucleic acid, a value which agrees well with the chemically determined figure of 1.0×10^{-9} mg. based on phosphorus estimations. Boivin & Vendrely (46) record a DNA content of 6.5×10^{-9} mg. per nucleus for liver, pancreas, kidney,

and thymus cells, while for bull spermatozoa the content was 3.4×10^{-9} mg. DNA per nucleus corresponding to the haploid number of chromosomes. The nucleic acid content of the yeast cell has been reported as 0.75 to 1.79×10^{-9} mg. per cell (42).

Tables showing the concentrations of both types of nucleic acid in different tissues have appeared in papers by Davidson (47, 48), Schneider (44, 49), Rerabek (50), and Euler & Hahn (51, 52, 53).

The nucleic acid content of the thyroid gland has been discussed by Rerabek & Rerabek (54), and of green leaves by Euler & Hahn (55). Human liver tissue has been examined by Friedman & Angrist (56).

The liver is of particular interest since most experiments on the effects of physiological and pathological changes on nucleic acid concentration have been made with this tissue, and good correlation has been found between the results of cytological examination and those of chemical analyses. A decrease in PNA content without alteration in DNA content, accompanied by a loss of basophilic cytoplasm granules, follows fasting (47) or the administration of protein-poor diets (57). Similar changes result from ischaemia. Ligation of the pedicle of one lobe of the liver of the mouse was found by Drochmans (58) to cause disappearance of cytoplasmic basophilia accompanied by a decrease in PNA content without change in DNA content. Administration of purified adrenocorticotrophic extract to rats on a high carbohydrate diet also caused a reduction in cytoplasmic ribonucleoprotein (59). Thymectomy does not affect the nucleic acid content of the liver (60). The effects of oral administration of nucleic acids have also been recorded (61).

The PNA concentration in the liver is significantly increased during pregnancy (47, 62). The DNA content is also raised and the total quantity found corresponds to the sum of maternal and foetal body weights (62). Campbell & Kosterlitz (63) have followed these changes at different stages of pregnancy in rats and find during the second week an increase in the amount of nuclear material and in its phosphorus turnover, while during the third week there is a marked increase in PNA content. Both PNA and DNA returned almost to normal values during the first week of lactation.

Administration of carbon tetrachloride to rats on a protein-free

diet or on a diet containing 18 per cent or 54 per cent casein as the protein source was followed by an increase in liver PNA interpreted as representing the formation of new liver cells in which the amount of cytoplasm was determined by the protein content of the diet (64).

In regenerating liver after partial hepatectomy the PNA content is markedly increased, reaching a maximum in one and one-half to three days after operation at a time when most rapid growth is found (65, 66). Although mitotic activity is most active at this stage, no consistent correlation could be found between the rate of growth and changes in DNA concentration. Increased succinoxidase activity accompanied the rise in PNA. The effects of protein free and high protein diets on the composition of regenerating liver were studied by Drabkin (67). PNA production was relatively independent of the dietary protein, but was apparently greater in the regenerating liver tissue from rats on a protein free diet than from those on a high protein diet. This has led to the suggestion that PNA, which is particularly concerned with protein synthesis during regeneration, is preferentially produced or "deposited" in such tissues as regenerating liver.

The nucleic acids in embryonic tissues have been reviewed by Brachet (68, 69). In the chick embryo Novikoff & Potter (70) found that the DNA concentration on a wet tissue basis rose to a maximum on the fifteenth day and then fell sharply. The PNA concentration fell from a high level on the second day to a low one on the fifth; it then rose to a maximum on the fourteenth day and fell off sharply. It is not clear which organs are involved in these changes but it would not appear to be either heart or liver since Davidson & Leslie (71) found no marked increase in either PNA or DNA in heart or liver at the fourteenth or fifteenth day. In cardiac tissue, the PNA concentration fell steadily from the ninth to the twentieth day while the DNA rose slightly. In liver both PNA and DNA concentrations fell during the incubation period. In chick embryo brain tissue (72) nucleic acids increased less rapidly than other constituents from the tenth to the nineteenth day of incubation. Further studies have been made in the nucleic acid content of embryo chick heart fibroblasts growing in tissue culture (73, 74).

The nucleoprotein content of nervous tissue has been extensively studied (75 to 78). Hydén & Hartelius (79) have shown that

the pentosenucleic acid content of ganglion cells in the central nervous system (C.N.S.) is increased by administration of small doses of malononitrile and have made use of this observation in the treatment of certain psychic disorders by malononitrile therapy.

CYTOCHEMISTRY

The ribonuclease test first described by Brachet (80) may be used in conjunction either with staining with basic dyes or with ultraviolet absorption (47, 81, 82). Its main defect lies in the possible contamination of the crystalline ribonuclease with proteolytic enzymes and the results must therefore be interpreted with caution and the test used preferably for confirmatory purposes only (47, 83). McDonald (84), however, reports that ribonuclease absolutely free from proteolytic contamination removes pyronophilic material both from cytoplasm and nucleolus. Stowell & Zorzoli (83) have made an exhaustive examination of the optimal conditions for the test with reference to type of fixative, type of buffer, temperature, concentration of enzyme, and period of incubation. They recommend McIlvaine's citric acid disodium phosphate buffer. Veronal acetate extracts considerable cytoplasmic material (83) from tissue sections, especially after acetone fixation (47).

The importance of the choice of a suitable fixative is also stressed by Tulasne & Vendrely (85, 86), who have used the ribonuclease test for the removal of PNA from bacteria in order to demonstrate the presence of "bacterial nuclei."

Sanders (87) has recommended celestine blue in preference to methyl green for use in conjunction with pyronine in the ribonuclease test.

Snapper *et al.* (82) have used the ribonuclease test in conjunction with lanthanum precipitation of nucleic acids to demonstrate that the basophilic inclusion bodies which appear in the cytoplasm of marrow cells in cases of multiple myelomatosis treated with stilbamidine consist of PNA. The conclusion has been verified by ultraviolet microscopy.

The ribonuclease test has also been used in the extensive histochemical investigations of Wislocki & Dempsey on the distribution of cytoplasmic nucleoproteins in the pineal body and pituitary (88, 89), the sweat glands (90), the placenta (88, 91, 92), the mammary gland (93), and in haemopoietic tissues (94, 95). In such

tissues the cytoplasmic basophilia of megakaryocytes (94) and of erythroblasts (96) is destroyed by ribonuclease.

An extensive examination of the cytological distribution of nucleic acids in haemopoietic tissues is reported by Davidson, Leslie & White (97, 98, 99), who found good correlation between the results of histochemical examination and figures obtained by direct chemical analysis of sternal marrow. PNA was located abundantly in the cytoplasm and nucleoli of the younger free cells and the amount diminished progressively with maturation. In pernicious anaemia the young megaloblasts with slight or no haemoglobinization possessed abundant basophilic material in their cytoplasm and the PNA concentration of the marrow was markedly raised above the normal level. The DNA was also raised owing to predominance of partially mature cells. Administration of liver extract or folic acid brought about increased maturity of the marrow associated with a fall in both nucleic acids to within the normal range. The fall was most marked during the reticulocyte response and was associated with the maturation of primitive cells possessing large nucleoli and basophilic cytoplasm and with the disappearance of megaloblasts.

The abnormal PNA content of haemopoietic cells in pernicious anaemia has also been observed by Thorell (100), who has made an exhaustive study by the Caspersson technique of the mechanism of formation of polynucleotides and proteins during blood cell maturation. Greatly increased metabolic activity of PNA with formation of cellular proteins was observed in leukaemia.

The occurrence of PNA in the nucleolus has been demonstrated with the aid of the ribonuclease test, since treatment with the enzyme reduces nucleolar basophilia (80, 99, 101, 102) and removes ultraviolet-absorbing material (47, 81).

In contrast to the ribonuclease test, the corresponding histochemical test employing desoxyribonuclease has been used only by a few authors (103). Desoxyribonuclease purified by McCarty's method (104) has been used to demonstrate the removal of DNA from chromosomes (105) and from the nuclei of the cells in sections of nervous tissue (87) or liver tissue (47). Here again the choice of fixative is important and chilled acetone is recommended (87, 47). Even so, results with this histochemical technique are not very reliable.

A new histochemical test for both PNA and DNA has been described by Turchini (284).

The Feulgen reaction.—Stedman & Stedman (106) have continued their vigorous criticism of the orthodox or conventional interpretation of the Feulgen reaction. They claim that the dye produced by the interaction of DNA and the fuchsin reagent is water-soluble and is avidly adsorbed by the protein chromosomin. Although the dye is produced only in the presence of DNA, structures stained by it consist rather of chromosin than of DNA which, in any case, is destroyed in the hydrolysis procedure. In support of the argument they have demonstrated (107) that isolated cell nuclei fixed with acid-alcohol and treated with warm hydrochloric acid lose all their histone and a large proportion of the phosphorus originally present. On the other hand Di Stefano (108), as the result of measurements of base, sugar, and phosphoric acid, has concluded that at optimum hydrolysis time in the usual Feulgen procedure one half the total base content has been removed from the nucleic acid, leaving a residue which is presumably the stained material. Postoptimal hydrolysis, however, causes removal of all the nucleic acid from the nuclei. The use of the Feulgen reaction for the quantitative determination of DNA in tissue sections is discussed by Stowell (109) and by Di Stefano (108).

Michaelis (110) has contributed an important paper on the nature of the interaction of nucleic acids and nuclei with basic dyestuffs.

Cytoplasmic particles.—The problem of the composition of cytoplasmic particles has been attacked by two chief methods: (a) the isolation of the granules by differential centrifugation and (b) the study of their composition by histochemical technique. Most work has been concentrated on the liver cell.

The technique of differential centrifugation has been improved by Claude (111), who has separated the cytoplasmic material of liver cells into three main fractions: (a) a large granule fraction composed of elements approximately 0.5 to 2.0μ in diameter corresponding to the mitochondria and the liver secretory granules; (b) a microsome fraction composed of submicroscopic elements 80 to $150 m\mu$ in diameter corresponding to the chromophilic ground substance of the liver cell; and (c) a supernate fraction con-

taining the soluble components of the extract. Measurements of phosphorus distribution and ultraviolet absorption have led to the conclusion that practically all the pentosenucleoproteins extracted from the liver cell cytoplasm are sedimentable and occur in association with the large granules and the microsomes. The results of Schneider (112, 113) suggest that most of the PNA of the cytoplasm is to be found in the microsome fraction.

Chantrenne (114), on the other hand, using mouse liver tissue, maintains that the cytoplasmic granules can be separated by centrifugation into five different fractions differing in PNA and enzyme content, nucleic acid being more abundant in the smaller granules. The speed of sedimentation of the nucleic acid was found to vary with the duration of the run, the type of buffer employed, and the magnitude of the gravitational field.

In the usual procedure of homogenizing liver tissue in water or isotonic sodium chloride solutions, mitochondria lose some of their morphological characteristics. To overcome this difficulty a new technique for the isolation of morphologically intact mitochondria has now been described by Hogeboom, Schneider & Pallade (115), who homogenized liver tissue in 0.88 *M* sucrose before proceeding to centrifugation. The mitochondria so obtained could not be distinguished morphologically from the mitochondria seen inside the liver cell on microscopical examination and they showed the characteristic ability to take up Janus green stain in very low concentrations.

Submicroscopic particles about 100 $m\mu$ in diameter were also obtained after prolonged centrifugation. Nineteen per cent of the PNA of whole liver could be accounted for in the mitochondria and 50 per cent in the submicroscopic particles, which were particularly rich in nucleic acid (116). The sucrose technique was employed by Price, Miller & Miller (117) in examining the distribution of nucleic acids in the livers of rats fed *p*-dimethylaminoazobenzene. Ingestion of the carcinogen reduced the protein content of the large granules by 35 per cent, lowered the level of PNA in the large and small granules by about 40 per cent, and decreased the levels of riboflavin in the large granules and supernatant fluid by 45 per cent. When the diet was low in riboflavin ingestion of *p*-dimethylaminoazobenzene caused an increase of protein and DNA in the nuclear fraction by 37 and 29 per cent, respectively.

Schneider (118) found that the PNA contents of rat livers and hepatomas were essentially the same but the nucleic acid per milligram of dry material was higher in the large granule fraction and in the unfractionated residue (including microsomes) of the hepatomas than in normal liver. The DNA content of hepatomas was greater than that of normal liver owing to an increase in the number of cells in the tumour tissue.

The enzyme content of the cytoplasmic particles has been studied by several authors. Using Claude's technique (111) for the separation of fractions from rat liver cell cytoplasm, Hogeboom, Claude & Hotchkiss (119) have concluded that the cytochrome oxidase and succinoxidase enzyme systems are associated, probably exclusively, with the large granules, little, if any, enzyme activity being found in the microsomes.

Similar results have been obtained by Schneider (112, 113, 118), who also found that 50 per cent of the adenosinetriphosphatase activity of normal whole liver tissue was associated with the large granules and 30 per cent with the unfractionated residue (including microsomes).

The mitochondria isolated by Hogeboom, Schneider & Pallade (116) after homogenization of liver tissue in 0.88 *M* sucrose contained 65 to 82 per cent of the succinoxidase activity of the original homogenate, the remaining enzyme activity being present in the fraction containing nuclei and unbroken liver cells. These authors conclude that probably all the succinoxidase activity present in the entire cell is due to the mitochondria. On the other hand Omachi, Barnum & Glick (120) found that esterase activity was located predominately in the microsome fraction which could be subdivided into a "lipoprotein" fraction and a "nucleoprotein" fraction. The former displayed thrice the enzyme activity of the latter.

Since PNA is found in cytoplasmic particles which are endowed with succinoxidase activity, it has been suggested that the nucleic acid might act as part of the enzyme structure. Potter & Albaum (121) found that a crystalline preparation of ribonuclease inhibited succinoxidase activity in tissue homogenates, but Schneider (122) subsequently showed that the ribonuclease preparation used by these authors had considerable proteolytic activity. The association of proteolytic activity with many preparations of crystalline ribonuclease has been confirmed by Cohen (123) and by Klecz-

kowski (124). Hence inhibition of succinoxidase by ribonuclease may be ascribed rather to destruction of the enzyme by the associated protease than to removal of PNA as an integral part of the succinoxidase enzyme system. Indeed Schneider (122) has shown that some preparations of crystalline ribonuclease with low proteolytic activity could remove 85 to 90 per cent of the PNA from rat liver mitochondria without causing appreciable loss of succinoxidase activity, but he is careful to point out (113) that the small amount of PNA still remaining in large granules after ribonuclease treatment may be associated with succinoxidase activity. Hogeboom (125) succeeded in bringing into solution some of the succinoxidase activity associated with the large granules of liver cell cytoplasm. These solutions contained PNA which could only partially be separated from the enzyme without loss of activity (113).

The cytoplasmic particles have also been studied by cytochemical methods. The ribonuclease test discussed above, in which basophilic cytoplasmic material is removed from tissue sections by incubation with the enzyme, has been applied to liver tissue by a number of authors (47, 59, 62, 81, 96, 126, 127, 128). While the particles whose affinity for basic dyes is removed by ribonuclease are of a size corresponding to that of the mitochondria, it may well be that they consist in fact of aggregates of microsomes (128). This view is supported by Brenner (129), who submitted fresh rat liver tissue to high speed centrifugation before fixation for histological examination, including the ribonuclease test, and concluded that "in general cytoplasmic ribonucleoprotein exists in the form of submicroscopic complexes within the cell." While analytical data on isolated fractions of particles reveal that the microsomes account for a very large proportion of cytoplasmic ribonucleic acid (111), Brenner's contention that "the cytoplasmic ribonucleoprotein bears no relation to the mitochondria" is not supported by the previously mentioned results of Hogeboom, Schneider & Pallade (116) on isolated morphologically intact mitochondria.

The particles in the cytoplasm of the liver cell show the strong absorption of ultraviolet light at 2570 Å characteristic of the nucleic acids (81, 130, 131, 132). The absorbing material is removed by ribonuclease treatment (47, 81, 130).

Nucleus and chromosomes.—From isolated cell nuclei or from homogenized tissue after extraction of cytoplasmic material with

0.14 *M* NaCl, Mirsky & Pollister (133) have prepared a material termed "chromosin" which they regard as the essential nucleoprotein complex of the nucleus. The name unfortunately lends itself to confusion with the "chromosomin" of Stedman & Stedman (134), and Mirsky (135) has now agreed to abandon its use. "Chromosin" dissolves in *M* NaCl giving a highly viscous solution from which it may be precipitated in long threads on pouring into six volumes of water. It consists chemically of three components, nucleic acid (mainly DNA), histone, and a nonhistone tryptophane-containing protein which has been labeled TrPr. This protein is obviously very similar to, if not identical with, the chromosomin of Stedman & Stedman (106, 134, 136).

Stern *et al.* (137, 138), however, regard Mirsky's "chromosin" as an artefact consisting essentially of a mixture of free sodium thymonucleate and histone chloride, and they claim to have prepared the true nucleoprotein from thymus nuclei in its native state by extraction with water after inhibition of nucleodepolymerase with arsenate. Their material, which precipitates as a gel in 0.14 *M* NaCl and has a particle weight of the order of a million, has been named "genoprotein T." The adoption of still another new name has little to recommend it and, in any case, it is doubtful whether any real chemical significance can be attached either to "chromosin" or "genoprotein." The cell nucleus is not a homogeneous structure and the real chemical interest lies in the composition of the individual parts of the chromosomes. This aspect of the problem has now been attacked by Mirsky & Ris (139), who have described a procedure whereby cell nuclei can be disrupted either by prolonged treatment in a Waring mixer or by passage through a colloid mill. The chromatin threads released by this procedure are said to be morphologically identical with chromosomes. In this way isolated chromosomes have been prepared from fish and fowl erythrocytes, from thymus and from liver, in sufficient quantities for chemical analysis.

When lymphocyte chromosomes prepared in this way are placed in neutral *M* NaCl they disperse to form a highly viscous suspension which, on centrifuging at 18,000 to 19,000 r.p.m. for 1 to 2 hr., yields a tightly packed sediment and a slightly opalescent, highly viscous, supernatant. The latter consists largely of nucleohistone which constitutes 90 to 92 per cent of the mass of the chromosome (140) and practically all of the nucleic acid in

this nucleohistone is DNA. When the viscous nucleohistone solution is poured into six volumes of water, a fibrous precipitate is formed containing 45 per cent DNA and 55 per cent histone.

The sediment, insoluble in *M* NaCl, on microscopical examination shows itself as a mass of coiled threads resembling chromosomes but rather smaller. Mirsky & Ris (140) have termed them "residual chromosomes." They account for 8 to 10 per cent of the mass of the original chromosomes and show longitudinal differentiation into thicker tightly coiled heterochromatic sections and more loosely coiled euchromatic regions. The nucleic acid of the residual chromosomes represents only about 4 per cent of the total amount present in whole chromosomes and is mainly PNA. The PNA content of residual chromosomes is 7.5 to 14 per cent while DNA is present only to the extent of 1.5 to 2.6 per cent.

The original chromosomes stain intensely by the Feulgen procedure but the residual chromosomes only faintly, since most of the DNA has been removed in the form of soluble nucleohistone. With pyronine-methyl green the original chromosomes stain purple-blue and the residual chromosomes red. This is in accordance with their respective nucleic acid contents.

When residual chromosomes are treated with hot trichloroacetic acid, nucleic acid is removed leaving a "residual protein" containing 13 per cent nitrogen and 1.36 per cent tryptophane. Thymus histone contains 18 per cent nitrogen and 0.14 per cent tryptophane (140). The residual protein is considered to be identical with the nonhistone tryptophane-containing protein TrPr previously described as a constituent of "chromosin" by Mirsky & Pollister (133).

The relative proportions of these various constituents vary in chromosomes from different sources. The residual chromosome forms only 5 per cent of the whole chromosome in fish erythrocytes but 40 to 50 per cent in chromosomes from liver. Only 45 per cent of the liver chromosome consists of nucleohistone as against 90 per cent in lymphocyte chromosomes (135). The proportion of nucleic acid present as PNA is 12 per cent for liver chromosomes, 3 per cent for thymus chromosomes, and only 0.15 per cent for trout sperm (135). Photometric analysis of nuclei by Pollister & Ris (43) reveals similar differences in the PNA/DNA ratio in thymus and liver.

It is interesting to examine how these observations fit in with

the views of Stedman & Stedman, who have now given an exhaustive discussion of chromosomin (106). They consider that the cell nucleus consists essentially of nucleic acid, histone, and a nonhistone tryptophane-containing protein, chromosomin, and that the chromosomes consist essentially of spiral threads of chromosomin embedded in a nuclear sap composed mainly of DNA which also forms the spindle. This view of course conflicts with the orthodox interpretation of the cytologists but some confirmation is to be found in the observation by Pollister & Ris (43) that nucleic acid is abundant in spindle fibres and asters. It is clear, however, that there is no real fundamental conflict between the views of the Stedmans and those of Mirsky. Both maintain that the essential basis of the chromosome thread is a nonhistone protein—whether it is termed “chromosomin” or “residual protein” or “TrPr” is of secondary importance—which is presumably embedded in a nucleic acid or nucleohistone matrix.

Davidson & Laurie (141) have demonstrated by paper chromatography differences in amino acid composition between histones and the nonhistone protein of the nuclei of thymus and liver cells and fowl erythrocytes. They found that the nonhistone proteins from thymus and liver contained 0.76 per cent and 1.20 per cent of tryptophane respectively, while the histones contained negligible amounts. Mirsky & Ris (140), on the other hand, report 0.14 per cent of tryptophane in thymus histone while that from calf liver contained 0.4 per cent and other histones none at all. Although Stedman & Stedman (106) regard the presence of any tryptophane in a histone as indicative of contamination, they are, nevertheless, inclined to believe that histones from different sources may have different amino acid compositions and this view is supported by other authors (141, 142).

Modern techniques, particularly those involving photometric methods, have made possible the study of the nucleic acid content of the chromosomes during mitosis and meiosis (45, 143). In mitosis the DNA increases in prophase and in metaphase reaches a value twice that found in interphase nuclei. The relative proportions of PNA and DNA remain unchanged in mitosis (45). In meiosis DNA doubles in the first prophase but not in the second division. The relative amounts of PNA and DNA are unchanged except in the later spermatid where there is a marked decrease in PNA and in nonhistone protein.

The more purely cytological aspects of the chromosomes have also been extensively reviewed (144 to 148).

NUCLEIC ACIDS AND PROTEIN SYNTHESIS

It is by now a well-established fact that cells engaged in protein synthesis have a high PNA content.

The views of the Stockholm school on the relationship between nucleic acids and protein synthesis have been reviewed by Caspersen (149) and by Thorell (100, 150). Similar views have been put forward by Boivin & Vendrely (16) as the result of their investigations on microorganisms. Brachet (151, 152) has emphasised the presence in the cytoplasmic particles of PNA together with proteolytic and respiratory enzymes and has suggested that these particles are in fact the actual organs for protein synthesis. But although a high nucleic acid concentration and the ability to synthesise protein are found concurrently, the mechanism whereby the nucleic acids might participate in the synthetic process is still obscure. Arising from Lipmann's exposition (153) of the importance of phosphate bonds in energy transfer for synthetic processes including peptide-bond synthesis, Muller (13) has maintained that some of the nucleotide fractions of nucleic acid, or close derivatives of them, are fundamental in those varied processes of the cell where it is necessary to effect a transfer of energy from a substance or group of substances relatively rich in energy to one relatively poor, with resulting synthesis, mechanical work, secretion against osmotic pressure, of other form of potential energy storage. Often an energy-rich phosphate bond is used by the nucleotide for the transfer.

Spiegelman & Kamen (154, 155) have also suggested that nucleoproteins are "specific energy donors which make possible reactions leading to protein and enzyme synthesis." Although many biochemists may consider this hypothesis improbable on thermodynamical grounds, some of Spiegelman & Kamen's experimental results are of great importance. They have demonstrated, for instance, that cells grown in a medium containing P^{32} build up a store of labeled "nucleoprotein" phosphorus, i.e., acid-insoluble nonlipid phosphorus or protein-bound phosphorus, which remains relatively constant so long as the cells are maintained anaerobically in the presence of glucose alone. When ammonium salts are added so as to evoke synthesis of new protein or when the adaptive synthesis of a new enzyme is forced, P^{32} flows out of this "nucleoprotein" fraction. Agents such as sodium azide and dinitrophenol,

in concentrations which prevent enzyme formation and protein synthesis without inhibiting fermentation, also stop the flow of phosphate from this fraction (154 to 157).

It may be significant in this connection that phosphate-transferring enzymes are easily inactivated by mustard gas (or its nitrogen analogues) (158) which is known to react with nucleic acids (159) and nucleoproteins (160) and to produce mutations (161).

Phosphatase.—If protein synthesis does indeed involve phosphorylation reactions, new significance must be attached to the well-known association of phosphatase with nucleic acids both in the cytoplasm and the nucleus. With the development of histochemical methods for the localization of enzymes within the cell, it has become clear that phosphatase is particularly plentiful in cells engaged in protein synthesis (102, 162). Thus it is abundant in developing teeth (163) and hair (164, 165), in bone marrow cells (166), in the epithelial cells of the small intestine (167, 168, 169), in anterior horn cells during the recovery period of chromatolysis after axon section (170), in the mammary gland (93), in healing wounds (171), in the silk glands of caterpillars and spiders (172), and in certain viruses (173). In embryonic tissues (162, 174, 175, 176) the phosphatase reaction is weak during the early stages of embryonic development but becomes intense at the beginning of differentiation when synthesis of new protein at the expense of the yolk is active. In vitamin C deficient guinea pigs phosphatase activity and collagen formation are both defective (177). Bradfield (172) has suggested that alkaline phosphatase is concerned in the production of many fibrous proteins and this view is supported by Jeener (178).

The problem therefore arises as to whether the occurrence of phosphatase in protein synthesising cells can be linked up with the presence of nucleoproteins (102).

The association of phosphatase with cytoplasmic basophilia due to pentosenucleoproteins has been recorded for pancreas (179), mammary gland (93), salivary gland (88), the placenta (88, 91, 92, 180, 181), osteoblasts (102), yeast cells (182), and embryonic tissues (162, 174, 175, 176). Nor is this association confined to the cytoplasmic nucleoproteins. Phosphatase is abundant in the chromosomes (183, 184) and has been demonstrated in the bands of salivary gland chromosomes of *Drosophila* (185). Moreover, a

distinct correlation has been found between the phosphatase content of cell nuclei and the turnover rate of the DNA (186). According to Brachet (186) the tissue in which protein synthesis appears to be most rapid (intestinal mucosa) (187) is also particularly rich in nuclear phosphatase and has a very rapid turnover of DNA phosphorus (188, 189), as well as a high nucleic acid content. Nuclear phosphatase activity is known to increase in epidermal cells treated with carcinogens (190).

During the phase of dissolution of the pentosenucleoproteins of the Nissl granules of anterior horn cells undergoing chromatolysis following nerve section, phosphatase activity is very marked at the stage of axon regeneration (170). In regenerating skin after methylcholanthrene treatment a rise in alkaline phosphatase activity is accompanied by a rise in PNA content (190). Even more striking evidence has been obtained by Jeener (178), who injected oestradiol into castrated female mice and observed a marked increase in alkaline phosphatase activity in the cytoplasm of the cells of the vaginal wall accompanied by a pronounced increase in cytoplasmic PNA. Similar changes were found in the uterus, but it is noteworthy that the intensity of alkaline phosphatase activity increased only in those cells which synthesise fibrous proteins (myosin and keratin).

Mirsky's recent results on chromosome composition (135) show that the "residual chromosome" (p. 168) in which PNA predominates contains all of the alkaline phosphatase of the intact chromosome. When PNA is split from residual chromosomes leaving the chromosome structure otherwise intact no phosphatase is liberated but when the residual chromosome disintegrates in chromosome autolysis, alkaline phosphatase is released and dissolves in the surrounding medium.

Similarly Jeener (191) has shown that treatment of cell nuclei or cytoplasmic granules with 0.6 *M* KCl, followed by ultracentrifugation, causes a separation of the material into a soluble portion containing nucleic acid and an insoluble residue rich in phosphatase.

These considerations suggest that some correlation exists between the synthesis of proteins, particularly fibrous proteins, and the occurrence of phosphatase and nucleic acids both in the cytoplasm and in the nucleus. The PNA of the cytoplasmic granules has, of course, long been associated with protein synthesis, and it is

known from the work of Brachet and his colleagues that these granules contain phosphatase (190, 192). It remains to seek a possible role of phosphatase in the synthetic process (173, 186). The suggestion has been made by Brachet (186) that a solution is to be found in a connection between peptide-bond formation and phosphorylation. Thus the synthesis of hippuric acid (193) from benzoic acid and glycine and of *p*-amino hippuric acid (194) from *p*-aminobenzoic acid and glycine may apparently proceed by way of phosphorylated derivatives of benzoic acid and it is tempting to suggest, as Spiegelman has done, that nucleic acids might act as phosphate donors in reactions of this type and "funnel energy into the protein synthesizing mechanism" (157).

Metaphosphate.—More recent evidence suggests the possible participation of metaphosphate. In 1936 Macfarlane (195) described a material which she had obtained from yeast, containing iron, nucleic acid, and metaphosphoric acid. Its phosphorus content was 16 to 18 per cent, of which 80 to 90 per cent was hydrolyzed in 7 minutes in *N* hydrochloric acid at 100°. This material appeared to be similar to the "Plasminsäure" described by Kossel (196) in 1893. The occurrence of metaphosphate has also been described in *Aspergillus niger* (197).

Interest in this metaphosphate of yeast has now been revived, largely as the result of the work of Wiame (198 to 201). It was shown by Brachet & Jeener (202, 203) that yeast cells cultivated in a phosphate free medium and then transferred to a medium rich in phosphate, synthesise very large amounts of basophilic substance. During the synthesis of this material inorganic phosphate is taken up in large amounts while the much lower increase in pentose and purines is only such as could be accounted for by the synthesis of PNA during growth. The basophilic material on isolation was shown to contain 15 to 17 per cent of phosphorus and 8 per cent of nitrogen and appeared to be responsible for the intense metachromatic reaction of the yeast cells (201). Subsequent investigation (199) has shown that metaphosphate can be extracted from yeast cells and can be obtained by precipitation as the barium salt. In the cell it probably exists as a polymer of a hexametaphosphate unit (198). Sodium azide ($2.5 \times 10^{-1} M$) completely inhibits metaphosphate formation in yeast without affecting glucose fermentation (199). Wiame's results have been confirmed by Schmidt, Hecht & Thannhauser (204).

Experiments with P^{32} have now shown that there are at least two different kinds of metaphosphate in yeast. That which is extractable with trichloroacetic acid in the cold does not appear to be metabolically very active, whereas the metaphosphate fraction associated with proteinaceous material is one of the most highly active organic phosphates in the cell (157, 205, 206). Wiame's view is that the energy of its phosphate bonds may be utilized for protein synthesis, and he is supported by Lindegren (207) working with the volutin of yeast cells which he regards chemically as metaphosphate and not as PNA. Lindegren suggests that the volutin (metaphosphate) coats the euchromatic chromosome previous to division, and that only chromosomes so coated with volutin are capable of division.

BIOSYNTHESIS

The mechanism whereby nucleic acids are formed in the living cell continues to be studied with the aid of isotopes. The observation of Plentl & Schoenheimer (208) that labeled guanine when administered to animals is not incorporated in the nucleic acids of the tissues has been confirmed (209, 210). On the other hand Brown *et al.* (209, 210) found that adenine labeled with N^{15} in positions 1 and 3, when fed to rats, was incorporated into the tissue nucleic acids and the isotope was also found to a lesser extent in positions 1 and 3 in the guanine of the tissue nucleic acids. Adenine can thus act as the metabolic precursor of nucleic acid guanine without rupture of the purine ring. Small amounts of N^{15} were also found in the muscle ATP but not in the pyrimidines of the nucleic acids.

The suggestion has been made (211) that, in the pigeon at least, the pathway from ingested ammonia or amino acids to uric acid passes by way of the purines of the tissue nucleic acids. If this is so, it is clear that a study of the distribution of isotopes of nitrogen and carbon in the uric acid in the excreta of organisms to which compounds containing these isotopes have been administered, may yield information about the mechanism of the synthesis of the purines of the tissue nucleic acids.

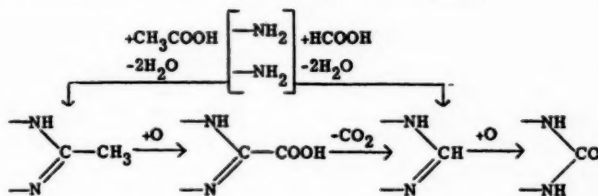
The formation *in vivo* of the carbon chain of uric acid, and therefore presumably also of the purines of the nucleic acids, has been studied with the aid of isotopic carbon (212, 213). Sonne, Buchanan & Delluva (212) administered to pigeons the com-

pounds $C^{13}O_2$, $HC^{13}OOH$, $CH_3C^{13}OOH$, $NH_2CH_2C^{13}OOH$, $CH_3CHOHC^{13}OOH$, and $C^{13}H_3C^{13}HOHCOOH$ and degraded the excreted uric acid by procedures which permitted the isolation of both the ureide carbon atoms (2 and 8) for isotope analysis.



They concluded that the carboxyl group of acetate and formate and the α - (or β -) carbon atom of lactate might participate in ureide synthesis but not the labeled carbon atoms of the other compounds studied.

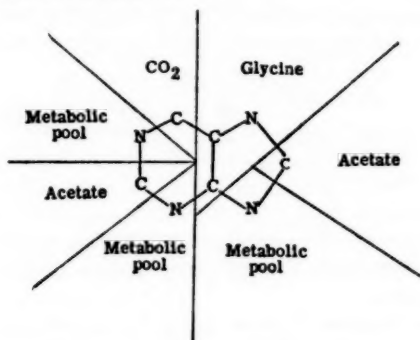
Acetate was suggested to act as the biological source of ureide carbon by linkage of its carboxyl group with nitrogen atoms to form a methyl substituted amidine structure thus:



Formate might either function as an intermediate in a similar way or might be readily converted into an intermediate. The isotopic carbon of α , β -labeled lactate is converted into ureide carbon since the carboxyl carbon of acetate can be derived metabolically from the α -carbon of lactate.

The source of the other carbon atoms in uric acid has also been studied by Buchanan, Sonne & Delluva (213). Carbon atom 6 is derived from carbon dioxide by an assimilation reaction hitherto undescribed. Glycine provides atoms 4, 5, and 7; its carboxyl carbon is the source of carbon 4 and its α -carbon is probably the source of carbon 5 while its amino group provides nitrogen 7. Shemin & Rittenberg (214), as the result of experiments on the excretion of uric acid in man, agree that glycine provides nitrogen 7 while nitrogen atoms 1, 3, and 9 come from the degradation of amino acids in general. Glycine has also been shown to be the source of nitrogen 7 in the guanine of nucleic acid of yeast (215).

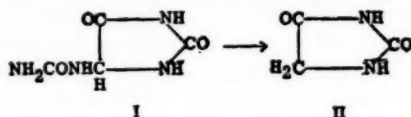
The source of the component atoms of the purine skeleton would therefore be as follows:



In *Lactobacillus casei* folic acid appears to influence the biosynthesis of purines and thymine (216).

It has already been mentioned that ingested labeled adenine is incorporated easily into the tissue nucleic acids but to a much smaller extent into muscle ATP (209, 210). Kalckar & Rittenberg (217) administered labeled ammonium citrate to rats and analyzed the adenylic acid subsequently recovered from the muscles. Since N^{15} had entered the amino group but not the ring, they concluded that muscle adenylic acid is subject to a rapid reversible deamination *in vivo*.

When ammonium salts or amino acids containing N^{15} are fed to pigeons the isotope is found in the tissue nucleic acids and in the excreted uric acid (211, 218), but in rats the isotope is found in the excreted allantoin (211), which is, of course, also labeled after administration of isotopic guanine (208) or adenine (210). After administration of adenine (210) or uric acid (219) labeled with N^{15} in positions 1 and 3 the excreted allantoin (I) was isolated and degraded to hydantoin (II) which was found to have the same

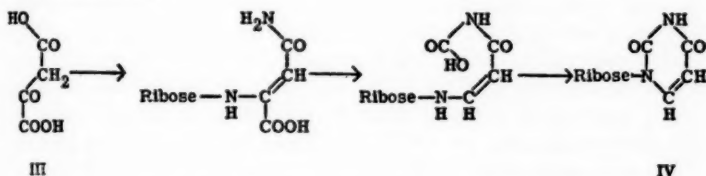


isotope content as the allantoin. Thus the N^{15} originally present in positions 1 and 3 of the purine ring must have become uniformly

distributed between the imidazole and urea moieties of the allantoin. This suggests that conversion of uric acid to allantoin involves the formation of a symmetrical intermediate such as hydroxyacetylenediureinecarboxylic acid (220).

When purines such as guanine or hypoxanthine are incubated with "ribose-1-phosphate" the corresponding nucleoside is synthesised and inorganic phosphate is liberated (221, 222). Whether such a reaction is involved in the coupling of purines and pentose in nucleic acid synthesis is, however, doubtful. It may well be that coupling with pentose precedes ring formation.

Thus, with the aid of artificially induced mutants of *Neurospora*, Mitchell & Houlahan (223, 224) have found that the carbon chain of pyrimidines arises from oxaloacetic acid (III). Uridine (IV) might be formed through intermediate aliphatic derivatives of ribose thus:



The source of the ribose is still not clear. The problem has been discussed by Gulland (225) and more recently by Cohen (226).

While the biosynthesis of individual parts of the nucleic acid molecule has thus been examined, attention has also been paid to the synthesis *in vivo* of the complete molecule of both types of nucleic acid. In a tissue containing few dividing cells the DNA is metabolically much less active than the PNA. Thus when P³² is administered to rats, the specific activity of the PNA phosphorus subsequently isolated from the tissues is much greater than that of the DNA phosphorus (189, 218, 227). Davidson & Raymond (218) fed labeled ammonium citrate to pigeons and rats and found appreciable amounts of N¹⁵ in the PNA subsequently isolated from the livers but only negligible amounts in the DNA. Brown, Peterman & Furst (228) fed labeled adenine to rats and found that in the PNA fraction of the viscera 15.9 per cent of the adenine and 9.1 per cent of the guanine originated from dietary adenine. In the DNA fraction the corresponding figures were only 0.55 per cent and 0.32 per cent. The ratio of PNA to DNA turnover is therefore

29.1 and, since this is higher than the corresponding figure for phosphorus turnover, they conclude that the phosphorus moieties in nucleic acid may be exchanged without purines being affected.

Although the DNA in resting cells does not readily take up P^{32} , the specific activity of the phosphorus is high in the DNA of rapidly dividing cells such as those of regenerating liver (47, 218, 227) and may even exceed that of the phosphorus in the PNA, as in embryonic tissue (47, 218). In tissues containing a high proportion of dividing cells, DNA is being rapidly synthesised and it is a matter of some interest to determine whether nuclear DNA can be synthesised directly from cytoplasmic PNA or whether, as appears much more probable, it is built up from smaller units.

Brachet (68, 69, 229) has maintained that in the early stages of embryonic development DNA is synthesised at the expense of cytoplasmic PNA by a conversion reaction of some sort, whereas in later stages both nucleic acids undergo total synthesis from unknown precursors. He has found that the developing sea urchin egg shows an increasing DNA content accompanied by a corresponding decrease in PNA (229). Schmidt, Hecht & Thannhauser (230), however, find that during the first 24 hr. of development of the *Arbacia* egg the amount of PNA per embryo remains practically unchanged whereas the DNA content increases steadily.

Mitchell (231 to 234) has described an accumulation of ultra-violet absorbing material in the cytoplasm of tumour cells exposed to x- and γ -radiations and suggests that the effect of radiation is to block a normal process of conversion of cytoplasmic pentose nucleotides to nuclear DNA. It is of course well known that rapidly dividing cells are especially rich in cytoplasmic PNA. Indeed the building up of a sufficient store of PNA seems to be a prerequisite for DNA synthesis. Davidson, Leslie & Waymouth (73) have found that chick heart fibroblasts growing in culture in a suitable nutrient medium show marked increases in PNA content, accompanied by cell migration several days before DNA begins to increase. This does not, however, necessarily mean that PNA acts directly as the precursor of DNA.

Cohen (235, 236, 237) has tried to settle the problem of DNA synthesis using the system *Escherichia coli* B infected with T_2 bacteriophage. This system synthesises desoxypentosenucleic acid only, in contrast to the normal bacterial cells which synthesise about three times as much PNA as DNA. In the infected cells pro-

tein and DNA were synthesised at a constant rate but while protein synthesis was apparent from the beginning of infection, DNA synthesis began 7 to 10 min. later. P^{32} studies revealed that the DNA phosphorus was derived mainly from the inorganic phosphate of the medium. PNA was inert in infected cells and did not appear as a precursor in the synthesis of DNA.

MICROORGANISMS

The nucleic acids of microorganisms have been reviewed by several authors (11, 238 to 241) and can only be briefly mentioned here. From yeast Chargaff & Zamenhof (242) have isolated highly polymerized DNA by a procedure avoiding drastic operations and enzymatic degradation. The PNA content of yeast has been discussed by Euler & Hahn (243).

Boivin (15, 244) has discussed in detail the directed mutation of *E. coli* under the influence of a factor consisting essentially of DNA. This process is analogous to the transformation of pneumococcal types described by Avery & McCarty (245, 246).

The ultraviolet spectrography of bacteria has been the subject of exhaustive studies by Malmgren & Heden (247 to 250), who have applied this method to the demonstration of the bacterial nucleus (251, 252) which has also been discussed by the Strasbourg group of workers (85, 86, 253 to 258).

Several reviews on virus nucleoproteins have appeared (237, 259, 260 to 262). Dawson & McFarlane (263) have studied vaccinia virus in the electron microscope. Crystalline pepsin removed the outer portions leaving a central body which could be digested away by desoxyribonuclease and probably corresponded to a nucleus.

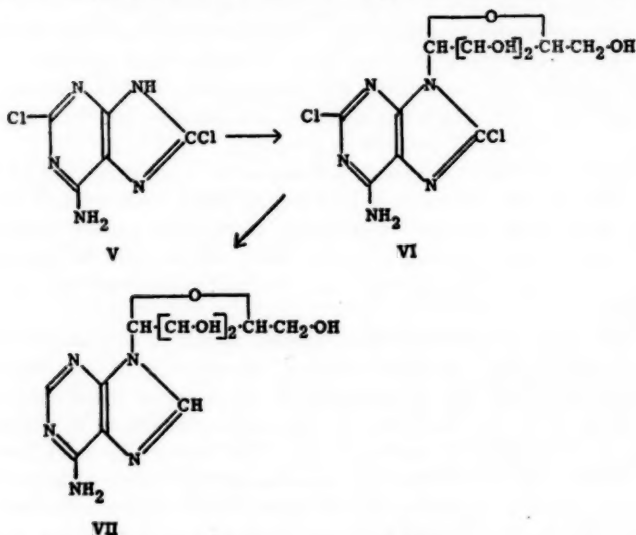
The virus of turnip yellow disease, which is transmitted by biting insects only, has been isolated and crystallized as a ribonucleoprotein (264). By centrifuging at high speed the virus was separated into two components, the upper consisting of protein only, and the lower of nucleoprotein. The former had much lower infectivity than the latter and was also less antigenic although it was not possible to distinguish between protein and nucleoprotein by immunological methods. The nucleic acid was separated from the nucleoprotein by treatment in the cold at pH 7 with alcohol, whereby the protein was denatured leaving the nucleic acid in solution. PNA prepared by this mild treatment could readily set

to a jelly and appeared to consist of large threadlike molecules. Physicochemical measurements on this virus (265) and also on tobacco necrosis virus (266) have also been recorded.

Cohen (235, 236, 237, 267, 268) has made important contributions to the study of the synthesis of bacterial viruses in infected cells.

NUCLEOTIDES AND NUCLEOSIDES

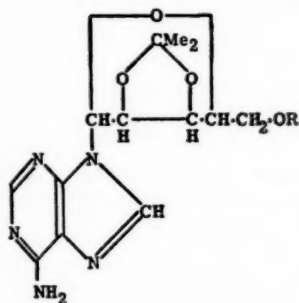
The structure and synthesis of nucleotides and nucleosides have been reviewed by Lythgoe & Todd (269). Their methods have now been extended to the synthesis of adenosine by condensing the silver salt of 2,8-dichloroadenine (V) with crude acetochloro-D-ribofuranose to yield a product which on deacetylation gave 2,8-dichloro-9- β -D-ribofuranosidoadenine (VI). Hydrogenation of this compound in aqueous sodium hydroxide in the presence of a palladinised barium sulphate catalyst yielded 9- β -D-ribofuranosidoadenine (VII) identical with naturally occurring adenosine (270).



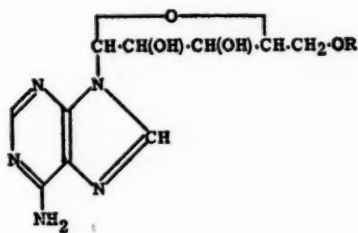
The structure and configuration of adenosine, and therefore of inosine also, have thus been definitely established. Guanosine has been synthesised in a similar manner (271) while cytidine has been

synthesised by allowing acetobromoribofuranose to react with 2,6-diethoxypyrimidine and treating the product with methanolic ammonia to yield 3- β -D-ribofuranosidocytosine identical with naturally occurring cytidine (272).

From the nucleosides the synthetic methods have been extended to the nucleotides (273). Treatment of 2',3'-*iso*-propylidene adenosine (VIII, R=H) with dibenzylchlorophosphonate yielded 2',3'-*iso*-propylidene adenosine-5'-dibenzyl phosphate (IX, R=PO(OBz)₂). Removal of benzyl groups by hydrogenolysis and of the acetone residue by mild acid treatment gave adenosine-5'-phosphate (X, R=PO₃H₂) identical with muscle adenylic acid. On the other hand, careful hydrolysis of (IX) with dilute acid under suitable conditions caused removal of the acetone residue with one benzyl group, yielding adenosine-5'-benzyl phosphate (XI, R=PO(OH)·OBz). When the silver salt of this compound was allowed to react with dibenzylchlorophosphonate in warm glacial acetic acid adenosine-5'-tribenzyl pyrophosphate (XII, R=PO(OBz)·O·PO(OBz)₂) was formed and on catalytic hydrogenation yielded adenosine-5'-pyrophosphate (XIII, R=PO(OH)·O·PO(OH)₂) isolated as the acridine salt which was identical with the acridine salt of natural ADP.



VIII-IX



X-XVI

The compound (XII) was not isolated but subsequent investigation revealed that crude preparations of this material contained appreciable amounts of a partially debenzylated material, adenosine-5'-dibenzylpyrophosphate (XIV, R=PO(OBz)·O·PO(OBz)OH), into which all the tribenzylpyrophosphate could be converted under suitable conditions. When the silver salt of (XIV) was allowed to react with dibenzylchlorophosphonate a yellowish

resin consisting largely of adenosine-5'-tetrabenzyl triphosphate (XV, $R = PO(OBz) \cdot O \cdot PO(OBz) \cdot O \cdot PO(OBz)_2$) was obtained which on hydrogenolysis gave adenosine-5'-triphosphate (XVI, $R = PO(OH) \cdot O \cdot PO(OH) \cdot O \cdot PO(OH)_2$) identical with naturally occurring ATP (274). Previous views on the structure of ATP have thus been confirmed.

Improved methods for the separation of the partial hydrolysis products of nucleic acids are now available. After hydrolysis of yeast PNA with 0.6 *N* barium hydroxide, Loring, Roll & Pierce (275) have described a method for the preparation of cytidylic acid and diammonium uridylate, involving fractionation of the phosphotungstates of the purine nucleotides and of cytidylic acid in weakly and strongly acid solutions under conditions in which uridylic acid is not precipitated. The pyrimidine nucleosides have been separated from hydrolysates of yeast PNA by means of the ion-exchange resin "Zeo-Karb 215" which retained cytidine but not uridine (276, 277).

The distribution of nucleotides in different parts of the hearts of various species has been described by Davies *et al.* (278). Adenine-pentose-pyrophosphate has been isolated from mung beans (279).

PURINES AND PYRIMIDINES

Methods have been described for the separation and identification of purines and pyrimidines involving counter-current distribution (280) and paper chromatography (6, 281, 282).

Adenine is responsible for the main part of the growth promoting power of PNA towards *Drosophila* although its growth stimulating action is regulated by guanine (285).

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METABOLISM OF THE LIPIDS¹

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ABSORPTION, DIGESTION, EXCRETION

Classical views on the mechanism of fat absorption continue to be challenged vigorously; the newer findings support at least some phases of the Frazer hypothesis (1). Zilversmit, Chaikoff & Entenman (2), using radioactive phosphorus as indicator, have reinvestigated the turnover of the phospholipids of the intestinal mucosa in fasted and fat-fed dogs and rats. They found that the turnover rates of phospholipid phosphorus, expressed as relative specific activity (ratio of specific activity of phospholipid phosphorus to specific activity of total acid-soluble phosphorus), of the mucosa of the duodenum, jejunum, and ileum of fasted normal dogs were nearly identical. In a series of fat-fed dogs (50 ml. of cream orally) the turnover was not only nearly identical in the three portions of the small intestine but did not differ from the average rates in the fasted dogs.

Experiments were also done on dogs in which the small intestine was ligated and fat (cream, corn oil, or corn oil fatty acids) was injected directly into the lumen of the duodenal or the ileal side of the ligature. Thus in the same dog the turnover rates of the phospholipid phosphorus in the fat-absorbing and the non-absorbing areas were examined and again found to be essentially identical. Phospholipids of the villi also showed no increase. It was concluded that fat absorption does not stimulate the rate of phospholipid turnover in the intestinal mucosa and, as a corollary, that phospholipids are not obligatory intermediates in fat absorption. Although previous work in Chaikoff's laboratory (3) had indicated that absorption of fat did increase the rate of turnover somewhat, as did the more recent work of Schmidt-Nielsen (4), it was felt that the differences could be ascribed to individual variation of animals and could by no means account for a stoichiometric relationship between amount of fat absorbed and amount of phospholipid turnover. As Zilversmit *et al.* point out, it is pos-

¹ This review covers the period from November, 1947 to November, 1948.

sible that glycerophosphorylcholine serves as a relatively stable skeleton to carry fatty acids and the use of radioactive phosphorus, of course, cannot test this question. The examination of fatty acid turnover in the phospholipid molecule during fat absorption, using labeled fatty acids, obviously should furnish some decisive information and the authors state that such work is in progress.

Artom & Swanson (5) have studied a related problem: the mechanism of absorption of phospholipids, which have generally been assumed to undergo intestinal hydrolysis prior to absorption. Labeled phospholipids obtained from livers of rats after injection of large doses of radioactive phosphorus as Na_2HPO_4 were administered as emulsions by stomach tube to fasted rats. Control rats were given either the same material following hydrolysis or unlabeled phospholipids plus an equivalent amount of inorganic radioactive phosphorus. The specific activities of plasma and liver phospholipids were measured after an absorptive period of three to six hours. After labeled phospholipid was fed the specific activity of the plasma phospholipid was some seven times as high as the activity after administration of the control mixtures. Although the data cannot be analyzed quantitatively due to the complexity of the turnover phenomena, it appears qualitatively that a portion of the ingested phospholipid may be absorbed as the intact molecule.

The views of Frazer (1) have also stimulated investigations on the promotion of fat absorption by emulsifying agents. Several groups have found that vitamin A absorption, which is probably a good indicator of fat absorption, is enhanced by such agents (6 to 10).

Heersma & Annegers studied quantitatively the effect of bile diversion on steatorrhea in dogs (11). In a further study (12) they found that various purified bile compounds (desoxycholic acid, etc.) in 3 gm. doses failed to reduce steatorrhea in bile-fistula dogs, although fresh ox bile was effective. Annegers and colleagues (13) feel that fecal fat excretion in normal human subjects is largely independent of dietary fat intake and that the fecal fat should not be regarded simply as undigested residue of ingested fat. Their views are not entirely in agreement with those of Wollaefer, Comfort & Osterberg (14).

Rapeseed oil, liquid at room temperature, has been found to have a very low coefficient of digestibility in rats; this is believed

to be due primarily to poor absorption of erucic acid (15). In a preliminary report, Morehouse, Cheng & Deuel (16) found that the inclusion of calcium and magnesium salts in the diet reduced the digestibility of a synthetic triglyceride (trilaurin, melting at 47°C., whereas these salts had no effect on the absorption of a blended lard (melting point 48°C.). Cook, Polgar & Thompson (17) have found a dextrorotatory acid of high molecular weight in the feces of rats on a high cholesterol diet, which apparently is derived from cholesterol.

Frazer has presented his views on fat absorption in general terms (18).

INTRAVENOUS ADMINISTRATION OF FAT

Considerable advances in perfecting the technique of preparing fat emulsions suitable for intravenous use have been reported from Stare's laboratory. Suitable experiments revealed that the granulomatous lesions and scarring previously observed after continuous administration of fat emulsions were due to the presence of toxic materials in the crude soybean phosphatides used as emulsifying agents. Geyer *et al.* (19) purified the crude material to obtain preparations showing no toxic manifestations. With the purified phosphatide, emulsions of fat could be given daily for 84 days to young dogs without evidence of lesions (20). Geyer and associates (21) also studied techniques of homogenization. Particle size of the emulsions was estimated by photomicrographic methods. Also described (22) was a micromethod for the determination of infused fat in blood by measuring the turbidity under standard conditions. The method was shown to give data linear with fat concentration within limits. Using this method, Geyer *et al.* studied fat tolerance curves after intravenous administration of test doses of emulsified coconut oil in the dog, rat, and rabbit. After administration of the test dose to the dog the fat level in the blood was about 1.0 gm. per 100 ml. Within two hours the level fell to about 0.2 gm. per 100 ml., indicating rapid deposition. Mineral oil emulsions were given intravenously and the turbidity of the serum followed in the same way. It was found that the mineral oil left the circulating blood much more slowly.

Meng & Freeman (23) have also made a thorough study of parenteral fat administration. Butter oil emulsions stabilized with soybean phosphatides, sodium cholate, or Span 20 were given

intravenously to dogs with no significant effects on blood pressure. Urine, bile, and thoracic duct lymph secretion increased somewhat. They observed no significant toxic effects of the emulsions after daily infusion for four weeks. Berry & Ivy (24) have given fasting dogs intravenous doses of 1 gm. of fatty acids per kg. body weight in the form of fatty chyle collected from cannulated thoracic ducts of donor dogs and also in the form of a 10 per cent emulsion of butter oil as prepared by Meng & Freeman (23). Blood fatty acids were then measured with the finding that the disappearance of fat from the blood was significantly more rapid when the fat was in the form of chyle than when it was given as a synthetic emulsion. This may have been due to a finer and more homogeneous dispersion of the fat in chyle.

Horlick, Feldman & Katz (25) have administered plasma from donor chickens on a high cholesterol diet (blood cholesterol of such animals is extremely high, frequently exceeding 2,000 mg. per 100 ml.) into normal chickens by the intravenous route and have obtained blood disappearance curves. The rate of removal of cholesterol was found to be a function of its concentration; normal levels were reached 12 to 24 hr. after injection.

TRANSPORT

There has been considerable interest in the turnover of the plasma phospholipids. Zilversmit, Entenman & Chaikoff (26) have determined turnover rates of plasma lecithin and sphingomyelin by measuring the rates of disappearance of the radioactivity of labeled phospholipids obtained from donor dogs treated with inorganic P^{32} . They found the lecithin fraction to be removed at a rate somewhat higher than that of sphingomyelin; since the ratio of lecithin to sphingomyelin in serum is 4 to 5:1, the total turnover rate of lecithin is over five times as great as that of sphingomyelin.

The turnover of plasma phospholipids in depancreatized dogs has been studied by two groups of investigators with substantially similar results. Chaikoff, Zilversmit & Entenman (27) have found that the rate of turnover of plasma phospholipids in depancreatized dogs is not increased above that of normal dogs, indicating that phospholipids are probably not agents for the extensive transport of fat from depots to liver in diabetes. Harper, Neal & Rogers (28) have studied the rate of removal from the blood of depancreatized dogs of intravenously administered P^{32} -labeled phospho-

lipid obtained from donor dogs. The rate of disappearance was found to be a function of the phospholipid concentration. The absolute rate of utilization of plasma phospholipid was slightly lower in the diabetic group and was not altered by administration of insulin and glucose. However, it was increased by feeding raw pancreas.

Zilversmit, Entenman & Chaikoff (29) have studied the effect of choline on plasma phospholipid turnover; this is discussed under "Lipotropic Agents." Balfour has studied plasma phospholipid formation in normal humans and in certain pathologies using P^{32} as a tracer (30).

Heller & Thayer (31) have examined the increase in blood fatty acids, cholesterol, and phospholipids in chickens and turkeys following the administration of synthetic estrogens. Handler (32) has followed the effect of thyroid activity on liver and plasma lipids of choline- and cystine-deficient rats. Hypothyroidism caused great increases in plasma phospholipids in both normal and choline-deficient rats, while thyroid feeding diminished plasma lipid levels only slightly.

Sinclair has made a careful analysis of the lecithin, cephalin, and sphingomyelin content of the blood sera of different species (33, 34). Human, dog, pig, and beef sera contain very little cephalin (3 to 8 per cent of the total phospholipids), whereas turkey serum contains up to 20 per cent of its phospholipid as cephalin. Pedersen (35) and Tayeau & Breton (36) have considered the structure of lipoprotein complexes of serum which presumably are involved in fat transport. Forbes *et al.* (37) have measured that portion of serum cholesterol not bound to serum proteins by determination of cholesterol extractable from lyophilized serum by chloroform under mild conditions. In normal serum only a small and constant percentage is "free"; in hypercholesterolemic rabbits, some diabetic patients, and all hypothyroid patients tested there was elevation of the readily extractable fraction.

DEPOSITION AND DISTRIBUTION

In an interesting preliminary note, Shapiro and associates (38) have reported that minced adipose tissue from the groin of rats when incubated *in vitro* with blood or serum, caused a 10 to 40 per cent decrease in the fatty acids of the medium providing the

adipose tissue was taken from rats starved to a 25 per cent loss in weight. Fat-laden adipose tissue or heated adipose tissue did not show the effect, which could also be inhibited by fluoride and cyanide but not by azide. Oxidation of the fat was ruled out due to the very low oxygen uptake. The findings are best interpreted as representing active penetration of fat into the tissue coupled to metabolic activities.

Bernhard & Korrodi (39) have found that human bone marrow fat strongly resembles that of the depots. Newlin & McCay have demonstrated that the bone marrow cavities are actually mobile storage depots for fat (40): the iodine number of marrow fat reflects that of the subcutaneous fat and the dietary fat.

Beadle, Wilder & Kraybill (41) have shown that rats on a diet containing linseed oil (as flaxseed) will deposit considerable amounts of trienoic acid in depot fat (up to about 25 per cent of the fatty acids present). The deposition of trienoic acid appears to be dependent on the amount in the diet. Samples of "yellow" hog fat have been found to contain considerable trienoic acids, presumably of dietary origin.

Hodge and his collaborators have continued their study of the lipids of the fasting mouse and present data on the total liver lipids and the lecithin and cephalin fractions (42, 43).

The abnormal deposition of blood lipids leading to atherosclerosis has received considerable attention in the past two years. Chaikoff *et al.* (44) have presented a complete report on the production of atheromatosis in the bird by administration of diethylstilbestrol. Implantation of pellets of the synthetic hormone causes a sustained and large increase of blood cholesterol, phospholipid, and neutral fat, and leads to atherosclerosis of the aorta resembling that which occurs spontaneously in this species. Cholesterol feeding also produces atherosclerosis and although the blood cholesterol level produced by these two methods is approximately the same the nature of the lipids deposited differs. After cholesterol feeding considerably more cholesterol is deposited than after estrogen treatment, which causes the formation of a deposit high in neutral fat and phospholipid. Horlick & Katz (45) have also studied atherosclerosis in estrogen-treated chickens on both normal and low-fat diets. A low-fat diet reduced the spontaneous incidence of the lesion in untreated birds, although the blood cholesterol levels on normal and low-fat diets were about equal. Horlick &

Havel (46) were unable to produce arteriosclerotic lesions in rats by feeding cholesterol and/or propylthiouracil (to inhibit thyroid function) although this regimen resulted in very high levels of blood cholesterol. The resistance of the rat is puzzling, since cholesterol feeding readily produces lesions in the guinea pig and rabbit. Kellner, Correll & Ladd (47) have found that the feeding of Tween 80 with cholesterol to rabbits promotes cholesterol absorption; this leads to higher blood cholesterol levels and an earlier and more severe atherosclerosis. Moreton (48) has again called attention to the size of lipid chylomicrons as a predisposing factor toward deposition of lipids in the arterial wall. He has found that particles of polyvinyl alcohol, methylcellulose, pectin, or gum acacia of about the same dimensions as large chylomicrons may also be deposited after intravenous infusion.

Oster has reviewed the subject of the distribution and metabolism of the higher fatty aldehydes (49).

DIETARY FACTORS

Essential fatty acids.—Deficiency of essential fatty acids has been produced in the past only in young animals. Barki and co-workers (50) have now described its production in the mature rat. After restricted feeding of a fat-free diet for some two months, mature rats were readily depleted. On *ad libitum* feeding of the fat-free diet after the depletion period the typical symptoms appeared; they were alleviated by corn oil or ethyl linoleate and sometimes disappeared spontaneously after a long period. Fraenkel & Blewett (51) have found that linoleic acid is a dietary factor necessary for normal formation of scales on the wings of the moth *Ephestia*; arachidonic acid has no effect on wing development but is necessary for the growth of larvae. Medes and collaborators report on the essential fatty acid content of rats on a fat-free and pyridoxine-free diet (52).

Bernhard (53) and the von Eulers (54) have prepared reviews on the essential fatty acids and their functions.

The "butter factor" question.—Deuel and his colleagues (55) have been unable to confirm the claim of Boer *et al.* (56) that vaccenic acid (*trans*-11-octadecenoic acid) is one of the acids of butter fat responsible for the superior nutritive value of butter fat compared to vegetable fats under special conditions. More recently, Boer *et al.* (57) have withdrawn their claim after finding

that spectroscopically pure vaccenic acid has no special nutritive effect and conclude that the growth-promoting properties of summer butter may be due to other trace materials.

Jack & Hinshaw (58), and Nath and associates (59) have been able to obtain fractions of butter fat which are superior to whole butter fat and other butter-fat fractions in the growth of rats. Heftmann (60) has found that the low molecular weight fatty acids of butter play no special nutritional role; however, under special conditions (low intake of vitamins A and D) natural butter fatty acids were superior to the fatty acids of partially hydrogenated butter. Scott & Verney have found that young rats prefer hydrogenated vegetable oil to butter fat, corn, or cottonseed oil in self-selection experiments (61).

The curious galactose-fat relationship described by the Wisconsin group (62, 63) and by Nieft & Deuel (64) has been examined further by Richter (65), who feels that galactose has a specific effect on fat utilization, rather than the reverse possibility suggested by the other investigators. Richter's conclusion is based on self-selection experiments in which small amounts of galactose greatly increased the survival time of rats offered oleomargarine.

Leichenger, Eisenberg & Carlson (66) have found no differences in growth rate and health between two large groups of institutionalized children over a two year period, during which oleomargarine and butter were given in table fat quantities and compared. Euler and associates found no difference between butter fat and fortified oleomargarine as dietary fat sources on the growth, fertility and longevity of rats (67). Westerlund, however, has reported some differences in calcium and phosphorus metabolism in groups of rats fed either oleomargarine or butter fat (68).

Lipotropic factors.—A number of interesting papers have appeared on the relationship of choline to phospholipid metabolism. McArthur, Lucas & Best (69) have demonstrated *in vivo* incorporation of the lipotropically active triethyl homologue of choline into the phospholipid molecule. Channon *et al.* (70) had previously recorded an unsuccessful attempt; analytical difficulties were the limiting factor. McArthur *et al.* feel that the lipotropic action of choline is, therefore, not simply a matter of labile methyl groups but may also be dependent on the configuration of the intact choline molecule. Platt & Porter (71) have found that administration of choline to rats treated with inorganic P^{32} caused an increase

in the specific activity of the liver lecithin fraction but had no effect on the activity of the cephalin fraction. Ethanolamine, which is not lipotropic (at least in the adult animal), caused an increase in the activity of the cephalin fraction. Artom & Cornatzer (72) have found that simultaneous administration of fat enhanced the stimulatory effect of choline on the specific activity of liver phospholipids in rats on a low-protein, low-fat diet. Fat alone had no effect. Extending their work, Artom & Cornatzer (73, 74) have also studied the specificity of the stimulatory effect of choline on phospholipid phosphorylation in the liver. Single doses of ethanolamine, monomethylethanolamine, dimethylethanolamine, choline, monoethyl-, diethyl-, and triethylethanolamine, diethanolamine, triethanolamine, and ethylamine all produced an increase in the specific activity of phospholipids in the livers of rats on a low-fat, low-protein diet. No stimulatory effect was noted after methylamine, dimethylamine, ammonium chloride, butter yellow, glutathione, DL-serine, or inositol.

It is clear from these findings and the previous work of Perlman *et al.* (75, 76) that lipotropic substances cause a stimulation of the incorporation of inorganic P^{32} into liver phospholipids. However, some nonlipotropic substances [see also (75, 76)] are also capable of this type of stimulation, and it remains to be seen whether the stimulatory effects observed in this type of short duration experiment are actually a reflection of lipotropic action. It would be of some interest to know whether some of these stimulatory agents may undergo direct incorporation into the phospholipid molecule, as has already been shown for arsenocholine (77) and for the triethyl homologue of choline (69). Platt & Porter explain stimulation of cephalin activity by the nonlipotropic ethanolamine on the basis of mass action effects (71). It is also conceivable that such stimulatory effects may be more directly due to increase of the specific activity of phospholipid precursors rather than a specific effect on incorporation of precursor into the phospholipid molecule [cf. Zilversmit *et al.* (78)].

Zilversmit, Entenman & Chaikoff (29) have localized the site of action of choline more closely. They found that, although the administration of choline causes an increase in the specific activity of choline-containing lipids in the liver and plasma, the calculated turnover rate of plasma phospholipid actually does not increase whereas the calculated turnover rate of liver phospholipid does

increase. The increase in specific activity of plasma phospholipids is due only to the fact that the liver phospholipids, precursors of the plasma phospholipids, have a higher specific activity after choline treatment. The authors conclude that choline does not act by increasing fat transport via plasma phospholipids but rather by stimulating phospholipid formation within the liver itself. Bollman, Flock & Berkson (79) have determined the absolute turnover rate of phospholipid phosphorus in the liver of the rat by two independent approaches with good agreement, using the inorganic phosphorus level of the liver as base line.

Ennor & Stocken (80) have found that the total acid-soluble phosphorus, the adenosinepolyphosphate fraction, and the phosphocreatine of guinea pig liver are increased when the livers are made fatty by administration of carbon tetrachloride. These findings were correlated with the high oxygen uptake and acetoacetate production *in vitro* of these tissues, and with the participation of adenine nucleotides in fatty acid oxidation. Campbell & Kosterlitz (81) have studied the effect of dietary protein on the turnover rate of phospholipid phosphorus and nucleic acid phosphorus in rat liver.

Rose, Machella & György have found that methionine is considerably less active than choline in preventing fatty livers in rats on a diet containing a pure amino acid mixture instead of casein (82). Shaffer & Critchfield (83) have found that methoxine (the methoxy analogue of methionine) is lipotropically active but is quite toxic to rats. Heppel, Porterfield & Peake (84) have demonstrated lipotropic effects of caffeine, theobromine, and theophylline at low dietary levels.

MacFarland & McHenry (85) have shown that the choline-resistant, inositol-responsive fatty liver, which has been intensively studied by this and other groups of investigators, can be produced readily in rats on a fat-free, high carbohydrate diet supplemented with biotin and folic acid and abnormally high amounts of other B vitamins. Inositol largely prevents the fatty livers. Biotin and folic acid did not produce the fatty livers when normal amounts of other B vitamins were given. These findings are considered to be explanatory of the effects of a beef liver fraction previously shown to be instrumental in producing this type of fatty liver. Drill & Loomis have also observed a complex nutritional situation leading to fatty livers (86). The pathogenesis of alipotropic fatty infiltration of the liver of rats has been followed (87).

Antifatty liver factor (AFL) of pancreas.—A lucid review of the present status of this factor has been prepared by Chaikoff & Entenman (88).

The pancreatic factor prepared according to directions of the California group has been claimed by Pavlov to show lipotropic activity in rats on an alipotropic diet (89) and on a high cholesterol diet (90). However, Canepa & Ivy (91) have tested the lipotropic effects of Dragstedt's lipocaic versus the AFL factor obtained by the methods of Chaikoff's group after subcutaneous administration to rats on a high fat, low protein diet. Although both these preparations were active orally in the depancreatized dog, Chaikoff's extract had no lipotropic effect subcutaneously in the rat, whereas Dragstedt's lipocaic under the same conditions largely prevented fat deposition in the liver. They conclude that these factors are not identical. However, these findings may possibly result from choline which may have been present in the lipocaic preparation used and may have no bearing on the biological activity of the AFL factor (88).

ENDOCRINES AND FAT METABOLISM

Bennett *et al.* (92) have observed a ketogenic effect of pure growth hormone and adrenocorticotrophic hormone on fasted normal rats, which was probably due to increased production of ketones. Pure lactogenic hormone had no effect on blood ketones. Clement (93) has also noted mobilization of fat after administration of pituitary extracts. Entenman, Chaikoff & Reichert (94) have found that thyroidectomy of dogs is followed by a fatty liver, even on a high protein diet, whereas hypophysectomy alone does not have this consequence. However, hypophysectomy enhances the effect of thyroidectomy. Such a deposition of liver fat can be completely prevented if the dogs are given 2 gm. of choline per day (95). Since the diet (high protein) does not cause fatty livers in normal dogs, it appears that removal of both glands interferes with availability or utilization of lipotropic factors in the diet or has increased the requirement. In contrast are the observations of Shipley, Chudzik & György (96) that thyroidectomy prevents fat infiltration in the livers of rats on a diet low in lipotropic factors. The latter group also examined the effects of surgical removal of other endocrine glands.

As mentioned before, Handler has studied the effects of thyroid activity on the liver and plasma lipids of choline- and cystine-

deficient rats (32). Marx, Meserve & Deuel (97) and Ershoff & Marx (98) have observed a protective effect of dietary cholesterol against thyrotoxicosis. Rapeseed oil meal fed at high levels to chicks produced thyroid enlargement similar to that given by thiouracil (99). Abelin & Klingler have studied the regulation of lipid metabolism by the thyroid as well as by insulin and the pituitary (100).

Janes & Prosser have found slight alleviation of diabetic symptoms in alloxan-treated rats on high fat diets (101). The effect of insulin on fatty acid synthesis (102) is noted elsewhere in this review. Stetten has discussed insulin and rates of certain metabolic processes, including fat metabolism (103).

Effects of estrogens on blood lipids and atheromatosis have already been pointed out elsewhere in this review. Variations in the cholesterol content of the adrenals following castration or androgen treatment of rats have been followed by Simonnet *et al.* (104).

INTERMEDIARY METABOLISM

Oxidation of fatty acids.—Lehnigier & Kennedy (105) have studied the cofactor requirements of enzymatic fatty acid oxidation in suspensions of saline-washed particulate material from rat liver. Treatment of this particulate material with water causes it to lose the ability to oxidize octanoate to acetoacetate in the presence of adenosinetriphosphate (ATP), magnesium ion, phosphate buffer and molecular oxygen. However, the activity can be fully restored by the addition of certain neutral salts or nonelectrolytes, such as sucrose or glucose, cytochrome-*c*, and small amounts of C₄-dicarboxylic acids. The salt effect, evident also in previous work by Leloir & Muñoz (106) and Potter (107), may be explained by the finding of Kennedy & Lehnigier (108) [confirmed by Schneider (109)] that the system of enzymes catalyzing fatty acid oxidation is localized in the mitochondria of the rat liver cell. These cytoplasmic bodies are morphologically sensitive to osmotic changes; however, purely osmotic effects do not explain all the facts. The requirement of C₄-dicarboxylic acids was shown to be not entirely a matter of providing oxaloacetate for condensation of two-carbon intermediates to yield tricarboxylic acid for entry into the Krebs tricarboxylic acid cycle; small amounts, catalytic in magnitude, appear to be necessary for the oxidation of octanoate to the two-carbon unit stage (105). It was also shown that the oxidation of

these small amounts of C_4 acid causes esterification of inorganic phosphate, suggesting that oxidation-coupled phosphorylation is necessary to "prime" the oxidation of fatty acid [cf. (106)]. However, ATP itself is not sufficient to prime the oxidation. The oxidation of fatty acid in this system also causes esterification of inorganic phosphate above that noted for the priming reaction. Acyl phosphates of the fatty acids do not appear to act as intermediates in the oxidation, contrary to previous suggestions (110), although they are capable of donating phosphate to the adenylic acid system. These findings now point to a basic similarity of properties of the fatty acid oxidation system in rat liver, guinea pig liver (106), rat heart muscle (111), and rabbit kidney and liver (112, 113).

Green and his colleagues have studied fatty acid oxidation in washed rabbit kidney and liver preparations [which they term the "cyclophorase system" (114)] (112, 113, 115). Their findings on the general properties of the enzyme system confirm and extend earlier studies of Leloir & Muñoz (106), Lehninger (105, 116), and other investigators. Extensive data on the oxidation of potential intermediates are reported. It is of interest that the rabbit enzyme preparations studied by this group do not oxidize the higher physiologically occurring fatty acids such as palmitic, stearic, and oleic acids.

An outstanding development in the work reported from Green's laboratory is the demonstration by Atchley (115) that valeric acid is β -oxidized to form propionic acid which accumulates due to the fact that the enzyme system does not attack propionate and that isocaproic acid is β -oxidized to isobutyric acid. The end products were identified by counter-current distribution techniques.

It has often been suggested that antiketogenesis by carbohydrate is due to substrate competition: carbohydrate, when available, being oxidized in preference to fat. Weinhouse, Millington & Freidman (117) have compared the rate of formation of acetoacetate and respiratory carbon dioxide from endogenous sources and from isotopically labeled fatty acids present as substrates in liver slices from fasted versus well-nourished rats. The presence of an ample supply of glycogen in the slice caused no diminution in the rate of fatty acid oxidation compared to slices from fasted rats, which were low in glycogen. Furthermore, the addition of pyruvate to slices from fasted rats did not cause any effective competition

with fatty acid oxidation. Their data show a tendency, increasing with chain length, for fatty acids to be oxidized in preference to carbohydrate. These findings would exclude such a simple substrate competition between carbohydrate and fatty acid in the liver as an explanation for antiketogenesis.

Kleinzeller & Bass have studied the oxidation of butyrate by a microorganism of the *Bacillus subtilis* group (118). Their data suggest α -oxidation. Bargoni has approached the study of palmitic acid oxidation in *E. coli* (119). Annau and his associates (120) have described experiments on the oxidation of higher fatty acids by liver slices, the respiratory quotient of which drops sharply in the presence of fatty acids although the increases in oxygen uptake are quite small. They have shown that natural or "synthetic" lipoproteins also cause the R.Q. of liver slices to decline (121, 122). Eperjessy & Zathureczky (123) found that ammonium ion as well as various amines and ethanolamine, also causes the R.Q. of liver slices to fall with an increased production of ketone bodies. The authors speculate on a relationship between the ketogenic action of ammonia and the mechanism of action of choline.

Verkade and his associates have presented further data on the ω -oxidation of fatty acids (124). Simultaneous administration of carbohydrate with a test dose of triundecylin caused greater excretion of undecanedioic acid in the urine than administration of triundecylin alone. Although it appears established that administration of fatty acids of intermediate chain length (C_8 to C_{11}) causes appearance of small amounts of corresponding dicarboxylic acids in the urine, the significance of this newer work as it pertains to physiological fatty acid oxidation is not clear in view of the tracer investigation of Bernhard (125).

Chaikoff and collaborators (126) have demonstrated that palmitic acid, labeled with C^{14} in the C_6 position, when administered to alloxan-diabetic rats caused excretion of glucose containing the isotope in such amounts that a process other than carbon dioxide fixation appeared to be involved in the incorporation (probably entry of two-carbon units from the fatty acid into the Krebs cycle, etc.).

An extensive series of papers has appeared concerning German work on the metabolism of synthetic fats and the general problems involved in the synthetic production of fats suitable for dietary purposes. The methods of synthesis have been outlined by Keil &

Schiller (127). Paraffin hydrocarbons obtained either from natural sources or synthetically were oxidized catalytically to form fatty acids which were then fractionated to yield a mixture of fatty acids having from 10 to 23 carbon atoms. These were then esterified with glycerol and refined. They contained, in addition to normal saturated acids of odd and even numbers of carbon atoms, small amounts of dicarboxylic acids, hydroxy acids, keto acids, and branched chain acids, and the problem in brief was to determine the metabolic behavior of these compounds. Appel *et al.* (128) have outlined the behavior of odd-carbon acids, which they found to be stored in fat depots. Milk fat from sheep which had been fed such fatty acids contained no odd carbon acids below C_{10} ; α -, β -, and γ -methyl acids were not stored but were eliminated as lower branched chain acids in the urine, together with unidentified derivatives. Such branched acids inhibited growth. Dicarboxylic acids were excreted in the urine. Thomas, Weitzel, and Flaschenträger and their colleagues have studied the excretion of succinic acid, an end product of ω -oxidation according to Verkade, after feeding various dicarboxylic acids, alkylated dicarboxylic acids, and synthetic fats (129 to 136). Keil (137) has made an interesting study of the *in vivo* metabolism of some branched chain acids. Delta-propyloctanoic acid yielded β -propylhexanoic acid in the urine; ϵ -propylnonanoic acid yielded γ -propylheptanoic acid; ζ -propyldecanoic acid yielded β -propylhexanoic acid; and γ -methyldecanoic acid yielded α - and γ -methylsebacic acids. In each case the metabolism is consistent with the β -oxidation hypothesis and, in the last instance, with β -oxidation following ω -oxidation. Thomas & Weitzel have reviewed this work and the German literature covering the period 1939 to 1946 (138).

The fatty acid dehydrogenase of mammalian liver which brings about 9,10-desaturation has been found by Burton to require diphosphopyridine nucleotide as coenzyme (139).

Leloir (140), Medes (141), and Ohlmeyer (142) have published reviews covering fatty acid oxidation. Breusch has also reviewed the biochemistry of fatty acid catabolism in detail (143). His review is especially comprehensive of earlier and related work not covered by recent reviews of this subject; however, the interpretation of the more recent advances is sometimes unwarrantedly categorical. Breusch suggests that α -, β -dehydrogenation, 9,10-dehydrogenation, and ω -oxidation may occur at the same enzyme

surface because of the possibility that the fatty acid chain may assume a coiled configuration in which the sites of oxidation mentioned may be spatially available to the same active center. This hypothesis did not appear particularly convincing to the reviewer after some experimentation with the Hirschfelder molecular models. There is at present no compelling reason to assume that the same enzyme system is involved in these three types of fatty acid oxidation.

The recent extensive work on lipoxidase is considered elsewhere in this volume.

Acetoacetate metabolism.—Krebs & Eggleston (144) have studied the aerobic metabolism of acetoacetate in heart preparations and their extensive analytical data are in consonance with the isotope tracer experiments of Buchanan *et al.* (145) and Weinhouse *et al.* (146) which showed that acetoacetate is largely metabolized through the tricarboxylic acid cycle. Although earlier data of Krebs & Eggleston (147) indicated that acetoacetate was not converted into citrate in any great amount anaerobically, Floyd, Medes & Weinhouse (146) have now shown that over half of the citrate arising anaerobically in kidney suspensions was derived from labeled acetoacetate. Aerobically, brain suspensions caused the formation of considerable endogenous citrate in addition to labeled citrate derived from labeled acetoacetate.

Soodak & Lipmann have demonstrated the enzymatic synthesis of acetoacetate from acetate in the presence of ATP and the same pigeon liver preparation which they found capable of acetylating sulfanilamide (148). Coenzyme A was required for this reaction. Since sulfanilamide is a competitive inhibitor of acetoacetate synthesis, the data suggest a common acetyl precursor. In this connection, Stadtman & Barker (149) have found that enzyme preparations from *Clostridium kluyveri*, the same organism used in Bornstein & Barker's study of fatty acid synthesis (150), catalyzed a phosphoroclastic cleavage of acetoacetate according to the equation:



The acetyl phosphate formed appeared to be identical with monoacetyl phosphate. Thus both the synthesis and cleavage of acetoacetate may involve the intervention of phosphate. The question of the reversibility of the phosphoroclastic cleavage will be of special

interest. Califano & Vilano have found ATP to exert a catalytic effect on oxidation of acetoacetate by suspensions of *E. coli* (151).

The finding of Breusch & Ulusoy (152) that the δ -lactone of triacetic acid is converted to acetoacetate by liver preparations has been more extensively examined in independent investigations. Witter *et al.* (153, 154) have described the synthesis of free triacetic acid and methods of analysis. These investigators have found that free triacetic acid is converted to one mole of acetoacetate and one of acetate in crude liver homogenates and by a purified enzyme. It is possible that the enzyme involved is identical with the "acyl-pyruvase" earlier described by Meister & Greenstein (155), which cleaves α , γ -diketo acids to form pyruvic acid and a fatty acid residue. Meister (156) has found that triacetic lactone undergoes two reactions in liver preparations, the first leading to hydrolytic cleavage of the lactone and the second to cleavage of free triacetic acid. The potential bearing of these findings on the mechanism of fatty acid oxidation is obvious; it would be of some interest to learn whether these reactions, apparently catalyzed by soluble enzymes, also take place in the particulate suspensions of mitochondria which catalyze fatty acid oxidation.

Weinhouse & Millington have demonstrated that tyrosine labeled with C^{14} in the β -carbon gave rise on incubation with liver slices (157) to acetoacetate labeled at the α -carbon, in agreement with the mechanism postulated by Edson (158).

Lackey, Bunde & Harris have shown that acetoacetate, β -hydroxybutyrate, and butyrate when administered intravenously to rats under pentobarbital anesthesia caused an increase in the glycogen content of the heart; concomitant increases in liver or skeletal muscle glycogen did not occur (159). These findings are of some interest in the light of present knowledge of fatty acid metabolism. Tidwell & Axelrod have studied blood sugar levels after acetoacetate administration (160). Heilesen, by comparing blood ketone levels and respiratory measurements of human subjects during muscular exercise, concluded that the musculature carries on metabolism of fatty acids directly and independently of liver-ketone production (161).

Metabolism of acetone via the tricarboxylic acid cycle was suggested by Polonovski & Valdiguie to account for metabolic disappearance of acetone (162). Kaplanskii & Shmerling demonstrated inhibition of α -amino acid formation in liver slices from

ammonium ion and pyruvate by the ketone bodies (163). They present some evidence suggesting the formation of β -aminobutyric acid from ammonium ion and acetoacetate in liver slices.

Acetate metabolism.—Perhaps the most interesting development has been the realization that monoacetyl phosphate is not the actual intermediate involved in the phosphoroclastic cleavage of pyruvate (164, 165, 166) and that a substance containing the acetyl radical and labile phosphate, but not identical with synthetic monoacetylphosphoric acid, is formed by reaction of ATP with acetate in *E. coli* extracts (166). Kaplan & Lipmann have presented preliminary evidence that the unknown intermediate may be an acetyl phosphate derivative which is convertible to acetyl phosphate by treatment with dilute acid (166). It appears that acetyl phosphate may be a phosphate donor rather than an acetylating agent whereas the unknown derivative may be primarily an acetylating agent. Details on these findings and also on the role of pantothenic acid in these reactions are considered elsewhere in this volume.

Weinhouse & Millington (167) have studied the metabolism of carboxyl-labeled acetate (C^{13}) by bakers' yeast. The isotope distribution and content of citrate and respiratory carbon dioxide formed indicated that the Krebs tricarboxylic acid cycle was involved, although the distribution of isotope suggested that an unsymmetrical compound, rather than citrate, is the true intermediate. It is of some interest that the calculated C^{13} content of the carboxyl groups of the C_4 -dicarboxylic acids was higher than could be accounted for on the basis of the reactions of the cycle. The authors point out that this finding can be accounted for on the basis of a side reaction involving end-to-end condensation of acetate to form succinate.

Lynen has studied the conversion of deuterioacetate into succinate via the tricarboxylic acid cycle in bakers' yeast (168). Although there was considerable endogenous formation of unlabeled succinate which complicated interpretation of the data, the amount of deuterium in the succinate isolated ruled out citrate as an intermediate, confirming the more decisive experiments of Weinhouse & Millington (167).

Goldinger, Lipton & Barron have found that acetate is utilized by bone marrow slices (169). Webb & Elliott (170) found that acetate, but not acetyl phosphate, accumulated when respiring

brain suspensions acted on pyruvate. Malonate, malate, and α -ketoglutarate increased formation of acetate; fluoroacetate did not do so.

Fat synthesis.—Important advances in the study of fatty acid synthesis have been made. Perhaps the most spectacular has been the report of Bloch & Kramer (102) that the synthesis of fatty acids in liver slices from labeled acetate is stimulated greatly by the presence of pyruvate, an effect which is in turn considerably enhanced by insulin. Insulin did not stimulate when glucose was substituted for pyruvate, and actually depressed synthesis with acetate alone. These findings are consonant with the conclusion of Stetten & Klein (171) that fatty acid synthesis is impaired in the diabetic animal and indicate that, in addition to the hexokinase reaction, insulin may control the metabolism of pyruvate and specifically its role in fatty acid synthesis.

An equally important contribution is the analysis of fatty acid synthesis in *Clostridium kluyveri* by Bornstein & Barker (150). This anaerobe does not ferment glucose or pyruvate but obtains energy from the simultaneous dissimilation of ethanol and acetate, propionate, or butyrate. In this process ethanol is oxidized to acetate with the formation of molecular hydrogen, and higher fatty acids are formed by condensation of the acetate formed with the fatty acid of the medium to form higher fatty acids. The energy derived from the oxidation of ethanol to acetate is more than enough to account for the endergonic condensation and reduction of acyl radicals to form fatty acid. The over-all process is therefore exergonic and provides energy for growth. The energetic coupling most likely involves phosphorylated intermediates; cell-free preparations of this organism were used by Stadtman & Barker (149) in their demonstration of both a phosphoroclastic cleavage of acetoacetate and the formation of acetyl phosphate by oxidation of acetaldehyde in the presence of inorganic phosphate. These bacterial preparations obviously furnish highly approachable material for the study of the details of fatty acid synthesis and oxidation.

Schubert & Armstrong (172) have demonstrated that bicarbonate labeled with C^{14} , administered to the intact rat, causes a very low degree of incorporation of C^{14} into fatty acids: the saturated fraction was more active than the unsaturated. The radioactivity appeared to be present in only the odd-carbon atoms. The

claim of the authors that this incorporation can be readily accounted for on the basis of known interrelationships of the tri-carboxylic acid cycle does not appear justified to the reviewer. Bernhard & Bullet (173), using deuterium as tracer, have found that an intensive synthesis of fatty acids occurs in the intestinal tissues of rats after a carbohydrate-rich diet, as well as after a protein-rich diet.

Shapiro & Wertheimer (174) have studied the *in vitro* incorporation of deuterium into fatty acids of minced adipose tissue of rats on incubation in a medium of serum enriched with deuterium oxide. The degrees of incorporation observed were very small and appear to the reviewer to be close enough to the limit of sensitivity of the techniques used to require some comment or qualification by the authors.

White & Werkman (175) have found that nonproliferating yeast cells will approximately double their fat content in from 24 to 48 hr. if acetate is the sole source of carbon under aerobic conditions. Dried or lyophilized cells did not show fat synthesis. Witter & Stotz (176) have also studied the conditions leading to fat synthesis in *Fusarium lysopersici*. This organism, as well as other *Fusaria*, have been studied in Nord's laboratory (177, 178) from the standpoint of fat synthesis and the metabolic relationship to this process of a pigment isolated from *F. solani* D₂ Purple. Raveux has studied fat synthesis in the mold *Sterigmatocystis nigra* (189) and has prepared an extensively documented review on fat synthesis by microorganisms (190).

Preliminary reports by Anker (179, 180) indicate an interesting difference in fatty acid synthesis in intact rats following administration of labeled pyruvic acid and pyruvamide, suggesting that the latter does not undergo simple hydrolysis but rather conversion to an active C₂-intermediate resembling acetate.

Popjak has found with the aid of P³² that phospholipids in the fetus are synthesized independently of those of the mother (181).

It has become increasingly evident that biotin plays a role in the biosynthesis of oleic acid. This interesting development is considered elsewhere in this volume.

Kleinzeller has prepared a review on the synthesis of fat (182).

REVIEWS

In addition to review material already cited, general reviews of fat metabolism have been prepared by Eckstein (183), Kermack

(184), and Tripp (185). Bloch has reviewed the use of isotopes in the study of lipid metabolism (186). The published *Transactions* of the Macy Foundation Conferences on Biological Antioxidants also contain considerable pertinent material (187). Hilditch has published a second edition of *The Chemical Constitution of Natural Fats* (188).

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CHEMISTRY OF NEOPLASTIC TISSUE

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The monograph *Biochemistry of Cancer* by Greenstein (1) is a very useful summary and one hopes that the author will be able to produce frequent new editions, as the subject grows. Cancer is being investigated and more intensively studied than ever before. The present review deals only with carcinogenesis and attempts at therapy of cancer, in which fields great advances have been made during the last few years. Work on tumour constituents and metabolism, and on the relation of viruses and hormones to cancer is not discussed.

Experimental carcinogenesis.—The problem of the origin of cancer is extremely interesting and has progressed so that hypotheses for the possible action of carcinogens have been advanced. The research in this field up to 1946 was reviewed by British authors (2) and by Lacassagne (3, 4). Hartwell's list of compounds (5) tested for carcinogenic activity is still invaluable and a second edition would be welcome.

The specific change of normal into malignant cells can be brought about by a wide variety of means. Many of the agents which have been used in the treatment of cancer are known to induce cancer either in animals or man and will induce mutations and damage cell nuclei. The relation of these actions, particularly in the case of carcinogenic hydrocarbons, has been discussed (6). The problem of "arsenic cancer" has also been reviewed (7). Many of the chemical carcinogens produce effects like those caused by radiations. Among the phenomena which appear to be interrelated and might come under the heading of radiomimetic effects are: (a) induction of neoplasia; (b) nuclear damage including inhibition of mitosis and breaking of chromosomes; (c) prolongation of life of leukaemic animals; and (d) production of mutations. The vesicants such as mustard gas and the nitrogen mustards, produce many of these radiomimetic effects (8). The effects may be due to similar underlying biochemical lesions.

Urethane.—Urethanes and particularly ethyl carbamate have been used as narcotics in animal experiments for a long time. Be-

cause they proposed to use urethane in experiments planned to measure the effect of x-rays on blood leucocytes, Hawkins & Murphy (9) carried out control experiments and found that urethane had a leucopenic action. Nettleship & Henshaw (10) noticed that urethane treatment of C3H mice increased the incidence of pulmonary adenomata. A survey of hypnotics with regard to possible causation of pulmonary tumours (11) showed that the incidence of pulmonary tumours was not increased by any of eleven different barbiturates, by chloral hydrate, paraldehyde, alcohol, urea, or sulphon methane derivatives. Tumours have been induced by isopropyl urethane, methylenediurethane, and ethylenediurethane (12). In later experiments (13) offspring of mice injected with urethane late in term had an increased incidence of pulmonary tumours.

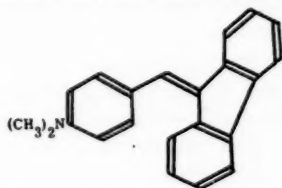
Some pathologists [e.g. (14)] consider that most of the tumours induced by urethane are benign adenomata, but there can be no doubt that some malignant growths have been induced and that the effect is specific.

Almost simultaneously with the discovery of the carcinogenic action of urethane in the United States, British investigators working independently (15) found that urethane had some beneficial effect in treatment of leukaemia. The discovery of the effect of urethane in human leukaemia followed on the observation (16) that the drug has some inhibitory action on experimental animal tumours. Many clinical reports on the use of urethane [e.g. (17)] have appeared and it has found application as an alternative to radiotherapy in the treatment of chronic myeloid leukaemia. Urethane treatment will prolong the life of mice with spontaneous (18) or transmissible (19) leukaemia and of rats with transmissible leukaemia (20). Urethane is a nuclear poison causing decreased mitotic activity in intestinal cells for 8 to 24 hr. after a single injection in mice (21, 22). More recent experiments (23) indicate that chromosome breaks in nuclei of the Walker carcinoma occur after giving doses of 1 gm. per kg. body weight to rats. About the same degree of nuclear damage is produced with a dose of 0.1 mg. of methyl di-(2-chloroethyl)-amine nitrogen mustard (HN2) per kg. This would suggest that the nitrogen mustard (HN2) is about 70,000 times more potent than urethane in producing this radio-mimetic effect. Taken in conjunction with other evidence [cf. (24)] the figures suggest that doses of 0.1 mg. of the nitrogen

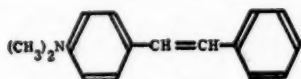
mustard (HN2) per kg., 7 gm. urethane per kg., and irradiation by 60 r are about equivalent in their effects on the chromosome material of tumours.

Methods are available for the estimation of urethane in body tissues and fluids (25, 26). In the latter method, which can be applied directly to blood, urethane is decomposed in Conway units with potassium hydroxide at 38°C. The liberated alcohol is absorbed in acid potassium dichromate which is reduced to an extent proportional to the urethane present in the sample. Work in progress at Birmingham, Alabama, with urethane containing C¹⁴ (27) should give indications of the possible mode of action of the drug as carcinogen or chemotherapeutic agent or even throw some light on the mystery of the action of narcotics.

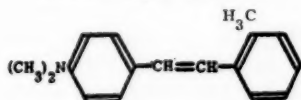
Aminostilbene derivatives.—An interesting group of carcinogens, from the point of view of growth inhibitory action, are the derivatives of aminostilbene investigated by Haddow *et al.* (28). Earlier experiments (29) had shown that growth of both normal and malignant tissues was inhibited by carcinogenic hydrocarbons. The association of growth inhibition and carcinogenic activity suggested that tumour production might be an adaptive reaction to specific interference with growth of normal cells. Following on the observation that 9-(4'-dimethylaminobenzylidene) fluorene (I) inhibited growth, the activity of 4-dimethylaminostilbene (II) was



I. 9-(4'-dimethylaminobenzylidene)fluorene



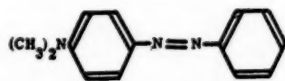
II. 4-dimethylaminostilbene



III. 4-dimethylamino-2'-methylstilbene

tested and found to produce marked inhibition of the growth of the Walker rat carcinoma. Of related substances 4-aminostilbene and 2-dimethylaminostilbene were active, but 3-dimethylaminostilbene was not active. Substitution of the α - or β -positions of the stilbene double bond destroyed the inhibitory action, but introduction of methyl or chlorine groups into the 2' or 3' position of the aromatic ring, as in 4-dimethylamino-2'-methylstilbene (III), increased the growth inhibitory action. On the other hand, substitution by any of a number of groups in the 4'-position resulted in disappearance of biological action. All the active compounds had the *trans* configuration in which the two benzene groups lie in one plane. Tests with *cis*-2'-chloro-4-dimethylaminostilbene, in which the two benzene rings are in different planes, showed it to be devoid of growth inhibitory action. Examination of the physical properties of these aminostilbene derivatives indicated that the active compounds were flat or planar molecules. Introduction of substituents which "buckle" the molecule reduces the biological activity, so that these substances resemble the carcinogenic hydrocarbons in that the molecules are flat.

After determination of the growth inhibitory action of aminostilbenes, tests for carcinogenic activity showed that 4-dimethylaminostilbene, 4-diethylaminostilbene, 4-dimethylamino-2'-methylstilbene and 1-(4'-dimethylaminophenyl)-2-(1'-naphthyl)-ethylene were carcinogenic. All these substances produced tumours at the site of injection but also remote tumours such as carcinomata of the acoustic duct, cholangiomata, and mammary adenomata. The incidence of tumours was similar to that which occurs on treatment with 2-acetylaminofluorene (30, 31). The close resemblance in molecular form between 4-dimethylaminostilbene and 4-dimethylaminoazobenzene [Butter Yellow (IV)] is worthy of notice.



IV. 4-dimethylaminoazobenzene

The inhibition of growth on treatment with 4-dimethylaminostilbenes does not occur if animals are fed on a high (20 per cent) protein diet. This is similar to the effect of a protein diet on growth inhibition by 1,2,5,6-dibenzanthracene (32). This effect of protein

in neutralising the inhibition of growth produced by carcinogens suggests that the carcinogens may function by interference with protein metabolism, and further study of the effect should give some indication of the mode of action of such growth inhibitors.

Azo compounds.—The specificity and complexity of the process of carcinogenicity is seen in the action of certain simple azo compounds. Several methyl derivatives of 4-aminoazobenzene are able to induce cancer of the liver, but tumours of the liver have been obtained with 4-aminoazobenzene itself fed to rats on a restricted diet when potatoes formed the source of starch (33). On a diet with rice starch, aminoazobenzene did not induce any hepatomas (34). There is no doubt that introduction of methyl groups into the molecule, as in dimethylaminoazobenzene and aminoazotoluene, increases the carcinogenicity (35). These carcinogenic azo compounds produce more definite biochemical changes in the liver and their effect is more readily influenced by diet than is carcinogenesis in other tissues by carcinogenic hydrocarbons. The carcinogenicity appears to be dependent on the concentration of riboflavin in the liver (36). Diets which accelerate tumour induction are low in this vitamin and the feeding of 4-dimethylaminoazobenzene reduces the concentration of the vitamin in the liver (37, 38). The effect of riboflavin in protecting rats from liver damage and tumour production can be considered established (39) but appears to be greater when the diet contains adequate protein. The high riboflavin diets may increase the ability of the liver to destroy the azo compounds by conversion to products which are no longer carcinogenic, but this does not appear to be the only way in which riboflavin exerts its anticarcinogenic influence. There is no evidence that the possible reduction products of azo compounds, such as aniline or alkylphenylenediamines, are carcinogenic. Aminoazo compounds increase the incidence of lung tumours in addition to hepatomas in mice (40).

The most interesting advance in this field is the discovery that bound aminoazo dyes occur in the livers of rats fed *p*-dimethylaminoazobenzene (41). The tissue proteins of the liver and blood plasma of treated animals are pink while the other tissues are not coloured and do not contain bound azo compound. Thus the azo compound is bound in the organ in which it induces tumours and the protein-azo compound is not found in the liver of the guinea pig or rabbit in which species the feeding of azo compounds does not induce hepatomas. The amount of bound azo compound was

greater in the livers of rats treated with dimethylaminoazobenzene on a low riboflavin diet which gave cirrhosis and liver tumours than in rats on a high riboflavin diet. *p*-Methylaminoazobenzene was taken up by the liver to about the same extent as the dimethylaminoazobenzene but *p*-aminoazobenzene was only bound to a slight extent. Examination of the livers of rats treated with aminoazobenzene and fed on a potato diet, on which hepatomas have been obtained, would be of interest. The results suggest that the ultimate carcinogen is probably a protein-azo-compound complex. A corresponding protein-carcinogenic hydrocarbon complex has frequently been postulated, but never demonstrated so clearly.

Choline-deficient diets.—The effect of diet on carcinogenesis is striking in animals treated with azo compounds, but animals maintained on choline-deficient diets appear to develop cancer in the absence of any extrinsic carcinogenic agent. Cirrhosis of the liver (42) may be produced in animals maintained on a low choline diet, and a high incidence of tumours of the liver was found in rats fed on a high fat diet with added cystine (43). Evidence that choline deficiency may increase the incidence of cancer has been reported by two different laboratories. In the Alabama laboratory (44), of 18 rats maintained on diets with 12 per cent or less of protein and 15 per cent lard, 14 developed neoplasia including growths in the lung, liver, pancreas, and abdomen. The published descriptions do not indicate how many of the neoplasias were malignant. Swiss workers (45) observed multiple adenomas of the liver in white rats maintained on a low choline diet.

The contribution of wave mechanics to the theory of carcinogenesis.—During the 10 years following the discovery by Sir Ernest Kennaway (46) of the carcinogenic action of 1,2,5,6-dibenzanthracene, carcinogenic activity has been found in some hundreds of chemical compounds. During the last few years a theory has developed which seems to make the carcinogenic action of hydrocarbons more comprehensible. An important and interesting characteristic feature of carcinogenic compounds is that they consist of flat planar molecules. The introduction of groups destroying planarity leads to loss of carcinogenic power. The electrons of molecules of hydrocarbons are either σ electrons, concerned in bonds between carbon atoms or between carbon and hydrogen, or π electrons, which are less firmly fixed in the molecule. In polycyclic hydrocarbons there are particular areas where π electrons

are concentrated, and Schmidt (47) was apparently the first to consider that the distribution of π electrons in hydrocarbon molecules might be one of a number of factors concerned in the activity of carcinogens. Methods of calculating the electron distribution in molecules, based on those of Pauling, were developed by Svart-holm (48). The French theoretical chemists R. and P. Daudel and A. and B. Pullman were encouraged by Lacassagne to develop these theories with reference to carcinogenesis (49) and in the opinion of the reviewer have developed a most interesting and fruitful theory.

A study of molecular diagrams (50) showed that each carcinogenic hydrocarbon molecule has an area of high "electron density." This area corresponds to the phenanthrene "double bond"; the French investigators named this the K region and calculated the charge on this region for a large number of derivatives of 1,2-benzanthracene and of benzacridines. In noncarcinogenic hydrocarbons the charge was less than 1.293e; introduction of substituents such as methyl groups increased both carcinogenic power and the electron charge of the K region. The theory is still being developed and methods of calculation of the electron distribution are being improved. The calculations are time consuming and the quantitative biological assessment of carcinogenic power is very crude.

The charge or density of electrons in a certain region or area of a molecule can be deduced by several different theoretical procedures. The simplest is that used by Pauling (51) in which the "double bond" character of different regions is calculated by consideration of the possible Kekulé formulae of the molecule. Other methods consider mesomeric molecular diagrams (52, 53), molecular orbitals (54), and "spin" (55). All methods give similar results. The different parts of the molecule are defined by the charge, bond order, and free valence. Biological effects appear to depend largely on bond order and free valence. The calculations show that the bond corresponding to the 9:10 bond of phenanthrene in more complex molecules should be particularly active and that 1, 2, 5, 6-dibenzanthracene has two such active regions. With the more elaborate and exact methods there is good agreement between the calculated charge and the carcinogenic activity as judged by mouse painting experiments. This holds with series of methyl-1,2-benzanthracenes, benzacridines, and 3, 4-benzphenanthrene de-

rivatives (56). Other carcinogens, such as aminostilbenes (57), have a region like the K region.

Between the theoretical calculations and the biological result is a great gap. The density or charge of a specific region of a molecule probably results in particular chemical activity. Substances with this kind of chemical reactivity in the presence of normal tissue tend to cause some normal cells to be transformed into malignant tissue. In order to bridge the gap in our knowledge the chemical reactions of carcinogenic and related substances are being studied. Although the most usual chemical reactions of aromatic compounds involve substitution, the reactions involved in carcinogenesis are most probably addition reactions. The elaboration of the hydrocarbon molecule necessary for carcinogenesis appears to increase the "double bond" character of a particular part of the molecule and so increase the probability of addition reactions occurring. Reactions of this type have been studied including (a) catalytic hydrogenation which is an addition reaction resulting in saturation of the bond of the K region (58); (b) oxidation with osmic acid in the presence of pyridine which yields complexes of dihydroxy compounds with the hydroxy groups in the "double bond" of phenanthrene (59), or in the K region of carcinogenic hydrocarbons (60): the rates of reaction in many cases increase with increase in carcinogenic activity particularly in any one series of compounds such as the methyl derivatives of 1, 2-benzanthracene (61, 62); (c) oxidation with perbenzoic acid, a slow addition reaction, the rate of which appears to vary with carcinogenic activity in a group of hydrocarbons which have been examined (64).

All the reactions which have been studied are in agreement with the theory that a K region or active double bond is of importance in carcinogenesis by hydrocarbons. If a chemical reaction could be found in which the rate or the equilibrium obtained were proportional to the carcinogenic activity it would add support to the theory, possibly give an easier way for determination of the activity and probably indicate the way in which these remarkable substances produce their effects.

Further support for the theory comes from the completely independent observations of Sir Robert Robinson (65), who pointed out that the common factor among the hundreds of carcinogenic hydrocarbons was a phenanthrene double bond activated by substituents in the molecule.

Time will show whether this interesting theory is correct or not. The French workers (66) have made predictions of the carcinogenic activity of a number of hitherto untested benzacridines and benzanthracenes by the mesomeric method. These substances are now being tested and the results will be compared with the predictions. At present the theory only deals with polycyclic carcinogenic compounds; it does not help to explain the action of urethane, of a low choline diet, or of various radiations. The relationships between structure, reactivity, and biological action of carcinogenic hydrocarbons have been reviewed (63).

The carcinogenic hydrocarbons probably form complexes with tissue constituents and such complexes remain in the organism for considerable periods. Work with 1,2,5,6-dibenzanthracene containing radioactive C^{14} (67) has shown that a small proportion of an injected carcinogen remains at the site of injection for many months. As there is always a considerable lag in carcinogenesis it seems probable that it is this persisting material which is responsible for the carcinogenic transformation. This lag between the application of a carcinogenic stimulus and the occurrence of the malignant change is a constant phenomenon for which we have no explanation. The lag is most clearly seen in tumour induction by irradiation.

A partial explanation for the delay in carcinogenesis by chemical agents is that some complex must act for a considerable period. This persistent complex is probably a protein-carcinogen compound. The experiments on inhibition of carcinogenesis with bromobenzene (68) and maleic acid (69), both of which react with thiol groups, suggest that the linkage is through sulphhydryl groups.

Metabolism of carcinogens.—The metabolic reaction of perhydroxylation by which polycyclic hydrocarbons are converted into dihydroxydihydro derivatives, which are called diols, was first demonstrated in anthracene metabolism. The same reaction has now been shown to occur in metabolism of naphthalene (70, 71) and phenanthrene (72). In the case of these simpler polycyclic compounds there are species differences; with the rat the products are laevorotatory while the rabbit metabolites are dextrorotatory, though often contaminated with racemic material. These diols are readily dehydrated to phenols by treatment with acid and it is possible that such diols are intermediates or precursors of the phenolic metabolism products which have been isolated in experiments with carcinogenic and other hydrocarbons. The fact that

rabbits are much more resistant than rats to the carcinogenic action of hydrocarbons may be associated with the difference in optical configuration of metabolism products produced by the two species.

The biochemistry of 3,4-benzpyrene was studied by the late Dr. F. Weigert using specially developed methods (73) in the intact animal (74) following intravenous injection and skin application, and also in isolated mouse skin (75). Although the metabolic products were not isolated they were characterised by chemical reactions, solubility, fluorescence spectra, and absorption spectra using ingenious micromethods. The first product seems to be a diol, 8, 9-dihydroxy-8, 9-dihydro-3, 4-benzpyrene or an ester of this compound. The fluorescent material "BPX" which is excreted in bile is probably a derivative of this kind. This diol or diolester is eventually changed in the small intestine to a phenolic compound 8-hydroxy-3,4-benzpyrene. Thus an early stage in metabolism of this carcinogen involves the reaction of "perhydroxylation" which has been found with simpler polycyclic compounds.

The experiments in which benzpyrene was painted on mouse skin (75) have shown that this reaction of perhydroxylation occurs in tissue of the skin and the diol BPX is the only derivative formed. This product then appears to diffuse away slowly. In other experiments the skin of recently killed mice was painted with benzpyrene and the isolated skin incubated in the Warburg apparatus for various times. At the end of the incubation the tissue was extracted and the metabolites estimated by physical methods. One point of great interest was that the metabolic diol did not diffuse out of either the intact or disintegrated tissue, although the diol itself is soluble in water. The diol was released by treatment with moist acetone. This indicates that the metabolite is combined with some nondiffusible tissue constituent, possibly protein. This complex is destroyed with moist acetone as some lipoproteins are broken up by solvents [e.g. (76)]. When 5 μ g. of benzpyrene was applied to 1 sq. cm. of detached shaved mouse skin quantities ranging up to 4 μ g. of BPX could be isolated after incubating for 6 hours. When one considers that the painting of benzpyrene on the skin of mice yields only tumours of the skin, this almost complete metabolism of the carcinogen in the skin seems of great importance.

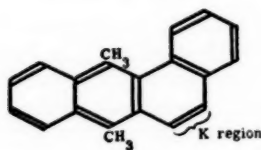
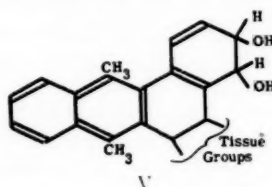
Studies with 1, 2, 5, 6-dibenzanthracene labelled in the 9 and 10 positions with C^{14} (77) show that when the carcinogen is injected

intravenously into mice 90 per cent of the radioactivity is excreted into the intestinal tract within 24 hours. The earlier findings that derivatives of carcinogenic hydrocarbons are excreted in bile was confirmed by the new technique. When the material was injected by the intraperitoneal route it became distributed in the body and was then slowly excreted. When the carcinogen was injected subcutaneously some of the radioactivity remained for long periods and amounts varying from 1.2 to 9.2 per cent of the originally injected material were found in tumours which appeared between $4\frac{1}{2}$ and 10 months after injection. Investigations of the chemical nature of the excretion products of 1,2,5,6-dibenzanthracene (78) show that only about 5 per cent of the carcinogen is excreted unchanged and about 3 per cent in a form which might be the phenolic dihydroxy-1,2,5,6-dibenzanthracene. A considerable fraction of the excretion products was acidic which indicates that a proportion of the hydrocarbon is extensively changed before excretion. A large proportion of the radioactive material remaining in tumours induced with radioactive 1,2,5,6-dibenzanthracene appeared to be unchanged hydrocarbon.

Another aspect of metabolism in relation to cancer is the possible formation of polycyclic carcinogenic hydrocarbons from hydroaromatic substances such as sterols. If cyclohexanecarboxylic acid is given to rabbits it is rapidly converted into benzoic acid which is excreted in urine (79). This reaction of aromatisation, that is, of cyclohexanecarboxylate to hippurate, has been observed with isolated slices of liver or kidney in the presence of oxygen (80). The reaction is inhibited by cyanide and also by the substrate, cyclohexanecarboxylate. If such a reaction accompanied by demethylation occurred in the body then a carcinogenic hydrocarbon might arise from natural tissue constituents.

Some metabolic processes undoubtedly lead to reduction of pharmacological activity and may be considered as "detoxication processes," but reasons for supposing that carcinogen metabolism and carcinogenic action are linked have been reviewed (6). Treating animals with agents which increase the rate of tumour induction, such as croton oil or cholesterol (81) or cholestanol (82), increases the rate of metabolism of 3,4-benzpyrene. The carcinogenic complex in the tissue may be a protein to which the carcinogen is bound through the K region or phenanthrene double bond and "perhydroxylated" in adjacent positions. In the case of 9,10-

dimethyl-1,2-benzanthracene the possible complex would be (V) formed from the hydrocarbon (VI). In all cases which have been studied the hydroxyl groups are introduced in positions adjacent to the K region. The linking of the K region may be through a



sulphydryl group. The facts (*a*) that the diols themselves are excreted, in the case of noncarcinogenic hydrocarbons, which have less activity in the K region and therefore a less stable linkage and (*b*) that the complex with the benzpyrene diol is decomposed with wet acetone suggest that the linkage is not very stable. It may be comparable with the combination of glutathione and maleic acid (83) and the stability would be expected to increase with increase in electron density of the carcinogen.

Carcinogens produce some of the effects of radiations and it is possible that the combination of a carcinogen with protein or the oxidation of the protein-carcinogen complex yields energy which causes the somatic mutation which finally results in cancer. If this were the case the radiomimetic effects would be due to production of specific effective radiations within the tissues. Study of the chemical oxidation of carcinogenic hydrocarbons has shown that the reaction produces visible light (84). The metabolic changes of carcinogens may be chemiluminescent yielding radiant energy which then causes damage to nuclear material. Another vague possibility is that the carcinogens which are fluorescent transform existing radiations either from tissue potassium or from cosmic rays into radiations which damage nuclei.

Cocarcinogens.—Analysis of the action of carcinogenic hydrocarbons on mouse skin has shown that the complex process can be divided into initiating and promoting stages. If a single application of 3, 4-benzpyrene is made to the skin of mice it does not induce cancer, but if the single application is followed by repeated treatment with croton oil, then tumours develop (85). The croton oil treatment alone will not induce tumours and so has only a promoting or cocarcinogenic action. In combined treatment with carcinogen and cocarcinogen the number of tumours which appear depends upon the activity of the carcinogen, but the latent period for tumour appearance depends upon the croton oil applications (86). If the croton oil is applied before the carcinogenic hydrocarbon then it does not affect the carcinogenic process (87); croton oil can only act after the initial step is completed.

These experiments show that carcinogenic substances may have at least two actions. The initiating stage is probably that which causes the nuclear damage and is similar to that produced by radiations. The cocarcinogenic action of croton oil on the skin is analogous to the effect of cholesterol in accelerating the rate of tumour induction by injected 3,4-benzpyrene (88). Cholesterol itself is possibly carcinogenic (89) and its feeble activity may be due to deficiency or absence of initiating action. The effect of croton oil is different from that of diets which increase carcinogenesis of aminoazobenzene derivatives; this effect of diet has been called procarcinogenesis (90).

Advance in our knowledge of carcinogenesis requires that the two factors of initiation and cocarcinogenic action should be defined. Some of the actions of methylcholanthrene, for example, may be those of an initiating agent while others may be due to a cocarcinogenic action. The more rapid action of methylcholanthrene as compared with 1,2,5,6-dibenzanthracene is thus possibly due to the former having cocarcinogenic activity.

Anticarcinogenesis.—The anticarcinogenic action of mustard gas (91) has been known for some years and the antagonistic action of bromobenzene (68) and maleic acid (69) on induction of skin tumours in mice by tar have already been mentioned. French workers have studied the antagonism of weak carcinogens toward potent carcinogens of similar structure (92). Thus painting of mice with the potent carcinogen methylcholanthrene produced tumours in an average time of 79 days, but when a mixture of methyl-

cholanthrene and the less carcinogenic 1,2,5,6-dibenzfluorene was applied, the average tumour induction time was increased to 108 days (93). In this experiment the dibenzfluorene appeared to block or impede the actively carcinogenic methylcholanthrene. Chrysene, which is not carcinogenic, had a similar antagonistic effect to dibenzfluorene; in other experiments (94) the carcinogenic action of 1,2,5,6-dibenzanthracene was reduced by simultaneous treatment with 1,2,5,6-dibenzacridine. The antagonistic effects are not likely to be due to chemical combination of the two substances used in each experiment. The antagonism may be due to the feeble or noncarcinogenic compounds becoming bound to the active centres in cells which would otherwise be free to take up the potent agent. Thus the carcinogenic action can be blocked by substances of related structure with less carcinogenic potency. The antagonism may be either on the initial or later stages of the carcinogenic process. The fact that the effect is mainly on the time of appearance of tumours rather than on the final tumour incidence suggests to the reviewer that the antagonism is on the cocarcinogenic part of the process.

Nitrogen mustards.—Our knowledge of the action of vesicants, including the nitrogen mustards, is largely a by-product of war: the damage to blood-forming organs by mustard gas [di-(2-chloroethyl)-sulphide] was described in 1918 (95). Similar damage is produced by the nitrogen mustards such as methyl di-(2-chloroethyl)-amine (referred to as HN2) (96) and tri-(2-chloroethyl)-amine (referred to as HN3). Mustard gas was shown to have an anticarcinogenic action in reducing the number of tumours produced by the application of coal tar to mice (91). Investigation of this anticarcinogenic action showed that mustard gas inhibited the glycolysis of tumour tissue (97). During the years 1940 to 1944 the pharmacology and biochemistry of mustard gas and nitrogen mustards were intensively studied in America (98), Belgium (99), and Great Britain (100) and many workers were impressed with the leucopenic and nuclear poisoning effects of the vesicants. The nitrogen mustards form hydrochlorides which are water soluble and nonvesicant and are therefore more suitable for clinical use than mustard gas. Because of their leucopenic action two of the nitrogen mustards, HN2 and HN3, were given by intravenous injection for therapy of Hodgkin's disease, lymphosarcoma, leukaemia, and disorders of the haemopoietic system (101). Pal-

liative effects with delay of the malignant processes have been most satisfactory in cases of Hodgkin's disease, although some cases of lymphosarcoma and chronic lymphatic leucaemia have also responded (102, 103). British workers obtained similar results (104, 105) using fewer but larger doses in treatment in order to reduce the amount of discomfort due to nausea and vomiting which follow treatment with these drugs.

This effect on white cells of the blood is a radiomimetic action and the nitrogen mustards are an alternative to radiotherapy in the treatment of lymphadenopathies. Some cases in which the disease has become radioresistant have responded to nitrogen mustard treatment. After the clinical results had been obtained these two nitrogen mustards (HN2 and HN3) were found to prolong the lives of animals with transmissible leucaemia (106) and with grafted lymphosarcomas (24). The nitrogen mustards have some beneficial effects on some cases of bronchogenic carcinoma (24, 102) but apparently not on cancers of other sites.

Mustard gas and the nitrogen mustards cause damage to chromosomes with inhibition of mitosis and production of chromosome breaks (107) similar to those produced by x-rays. These vesicants also resemble radiations in inducing mutations in *Drosophila* (108) and in *Neurospora* (109, 110). Carcinogenic hydrocarbons produce some of the effects of radiations including the induction of mutations in mice; this was demonstrated first with methylcholanthrene (111) and later with 1,2,5,6-dibenzanthracene (112). On account of the frequent association of tumour inhibitory activity, radiomimetic properties, and carcinogenic activity, nitrogen mustards were tested for carcinogenic activity (113). Mice were given weekly subcutaneous injections of HN2 and HN3. The hair at the site of injection became bleached in colour as it is when the skin of mice is irradiated [cf. (114)]. Among the mice which survived the treatment for a year lung tumours, lymphadenomata, and spindle-celled sarcomata occurred. This forms another example of a substance which inhibits growth, causes damage to cell nuclei, and induces tumours.

The typical lesion of the vesicants is a blister (a lesion also produced by radiations) and this appears to be preceded by a reduction in the hexokinase of the skin (115). When rabbits are poisoned with mustard gas the glycolysis of the bone marrow is very much reduced at about the time when histological abnormali-

ties appear in that tissue (116). Many British workers consider that hexokinase is particularly sensitive to the action of vesicants and that the pathological effects in tissues are due to the inhibition of this enzyme. Investigations carried out in Cori's laboratory have shown that not only hexokinase but the phosphokinases in general are sensitive to mustard gas and nitrogen mustards. The reviewer has suggested that the damage to nuclei which vesicants cause is due to inhibition of phosphokinases involved in nucleic acid metabolism within the nuclei. When rats bearing the Walker carcinoma are treated with a dose of HN2 (1 mg. per kg.), which inhibits tumour growth, a reduction of the glycolysis but not of respiration of the tumour tissue is seen about 4 days after dosing (24).

Nitrogen mustards also inhibit enzymes involved in choline metabolism (117). Thus choline oxidase is inhibited by 1×10^{-6} *M* solutions of HN2 or HN3. The inhibition is probably due to the action of the ethylene imonium form of the bases, $R-N^+ = (CH_2-CH_2)$. In this form the nitrogen mustards have convulsant and rapidly lethal actions (118); such effects might be due to inhibition of enzymes concerned with choline metabolism. The aliphatic chloroethylamines are extremely reactive substances, reacting with most cell constituents except unsubstituted sugars and fats (119), and it is not surprising that a variety of enzymes are inhibited by these agents (120). The inhibition of choline oxidase is partially neutralised by choline. The pyruvate oxidase system is also inhibited by vesicants, particularly by the arsenical vesicant, lewisite (121). The inhibitory effect of the active chloroethyl compounds on the pyruvate oxidase system is increased by diethyl and dihydroxyethyl thiocarbamates and it is difficult to understand how these compounds produce their effects (122).

From the clinical standpoint the nitrogen mustards are of possible value in the treatment of those cases of Hodgkin's disease and related conditions which do not respond to radiotherapy or in cases where the lesions are so widespread that radiotherapy is impossible. The disadvantages are the production of nausea and vomiting (possibly analogous to radiation sickness), shortly after injection, and of leucopenia and thrombocytopenia.

Aromatic chloroethylamines.—In extension of research on carcinogenic and growth inhibitory derivatives of 4-aminostilbene, a compound 4-N:N-di-(2-chloroethyl)-aminostilbene was prepared and examined and found to have remarkable growth in-

hibitory properties when tested against the Walker carcinoma. Following this observation a number of aromatic di-(2-chloroethyl)-amines have been synthesised (123) and examined for biological activity (124). The chloroethylamine derivatives of this series are much less toxic than the aliphatic nitrogen mustards; also the N:N-di-(2-chloroethyl) derivatives of aniline, *o*-, *m*-, and *p*-toluidine, *o*- and *p*-anisidine, β -naphthylamine, and *p*-aminobenzoic acid have marked inhibitory action, and have prolonged the lives of tumour-bearing animals.

These substances are of interest as they have some of the properties of nitrogen mustards but are much less toxic and are effective when administered by mouth. The mechanism of their action has been much less studied than that of the aliphatic analogues, but the 2-N:N-di-(2-chloroethyl)- β -naphthylamine has been found to cause a fall in the glycolysis of tumour tissue similar to that caused by HN2.

Radiations.—In addition to their use as tracers, radioactive isotopes have been studied from the point of view of therapy and from that of carcinogenesis. The results obtained in treatment of polycythaemia vera with P^{32} have been excellent (125, 126). In the treatment of some leucaemias administration of P^{32} is about as effective as the x-ray procedures (127), but in lymphadenopathies it appears to be inferior to normal radiotherapy. Reports on the use of I^{130} show that it is of value in treatment of hyperthyroidism (128, 129) and in some cases of adenocarcinoma of the thyroid (130).

Lacassagne has published a monograph on cancer produced by radiations (131) and a second monograph on carcinogenesis by radioactive materials (132).

The carcinogenic properties of radioactive fission products have been intensively studied during the last few years. The subcutaneous injection of 1 μ g. of plutonium or of 1 millicurie of yttrium phosphate induced fibrosarcomata in mice (133). Bone tumours were produced with plutonium and carcinoma of the colon with Y^{91} .

Studies of the tolerance range of x-rays (134) showed that the incidence of malignant lymphoma, lung tumours, and mammary carcinoma was increased by exposure of mice to 8.8 r daily over a period of many months.

Earlier work on the effects of radiation on tissue metabolism showed that tissue respiration was reduced on exposure of tumour

tissue to radium at 38°C. while the glycolytic power was destroyed on irradiation at 0°C. (135, 136). Recent work (137) suggests that radiations poison sulphhydryl enzymes by oxidation and has shown that phosphoglyceraldehyde dehydrogenase is inhibited by moderate doses of radiation. The inhibition is reduced if catalase is present. Many of the drugs which produce effects like radiations, such as the vesicants, can inactivate sulphhydryl enzymes. Experiments with rabbits (138) showed that the skin damage due to exposure to 2000 r was much less when the animal had a high blood sugar following injection of glucose, than when the blood sugar was normal. This protective action was attributed to the reducing action of the sugar, but other reducing substances such as glycine, glutathione, cysteine, or ascorbic acid had no protective effect (139). The protective action of the glucose may be due to the fact that hexokinase is less easily poisoned in the presence of high concentrations of its substrate, glucose. Thus the skin damage may be due to poisoning of hexokinase and is possibly analogous to the skin damage caused by vesicants.

Other experiments (140) also indicate that hexokinase is destroyed by γ -radiations. When tumour tissue was exposed to radium the ability of the cells to produce lactic acid from glucose was destroyed but the tissue could still produce lactic acid from hexosediphosphate.

Very little is known of the effects of combination of radiation and other carcinogenic agents; such experiments are difficult to interpret while the different parts of carcinogenesis are so indefinite. In studying the possible effects of cosmic radiation on carcinogenesis, mice injected with methylcholanthrene were kept under lead sheets (141). Mice under lead developed tumours more quickly than control mice in adjacent boxes which was attributed to the fact that the lead caused secondary showers; hence the mice under lead received more radiation than other mice. In this case the combined carcinogenic effect of radiation and methylcholanthrene was greater than that of methylcholanthrene alone.

Examination of the combined toxic actions of x-rays and nitrogen mustard (HN2) has shown (142) that if the HN2 is given first then the toxic effects are at least additive, while if radiation is followed by HN2 one or two hours later, the total toxicity is less than the sum of the two. This might be due to the radiation causing a temporary reversible change of essential tissue groups (perhaps

oxidation of sulphhydryl radicals) so that they cannot be attacked by the nitrogen mustard.

Quinones inhibit mitosis probably by inhibiting the prophase, although when given alone they do not cause rupture of chromosomes (143). They also appear to potentiate the effect of x-rays in causing nuclear damage; and 2-methyl-1,4-naphthohydroquinone-diphosphate has been used to potentiate x-rays in clinical therapy of cancer (144). In this case the quinone possibly catalyses chemical changes between activated water and constituents of cell nuclei.

The action of radiations on living cells was discussed by the late Dr. D. E. Lea (145), mainly from the point of view of the "target hypothesis."

The specificity of chemotherapeutic agents for cancer.—The therapeutic agents which have been so far described in this review are known to be carcinogenic. These agents are in general substances toxic to dividing cells and usually produce some damage in all growing tissues, probably by damaging the cell nuclei. Such substances increase the difficulties of cell division and may increase the probability of the "accident" of somatic mutation, thus leading to the development of cancer.

So long as nuclear poisons are used as chemotherapeutic agents the risk that the treatment may itself induce new tumours may be present. The search for agents which will inhibit tumour growth is difficult and if one is limited to agents which are noncarcinogenic the task is even more difficult. Such agents might possibly be found in cytoplasmic poisons with specific affinity for tumour cells.

The chemotherapeutic agents which are available all show some specificity for cancers of different sites. Thus oestrogens are of value in cancer of the prostate (146) and a few cases of breast cancer in elderly women (147); testosterone gives responses in some young patients suffering from breast cancer (148). Urethane has a palliative action in leukaemia, the nitrogen mustards in Hodgkin's disease and related conditions, and 9, 10-dimethyl-1, 2-benzanthracene in leukaemia (149). This specificity shows that drugs can differentiate between different types of tumour and so the differentiation between normal and malignant tissues should be feasible.

The groups of substances discussed below are not known to be carcinogens but that may be because they may not have been tested for carcinogenic activity.

Bacterial products.—Great progress has been made in the study of bacterial polysaccharides since the discovery, made in 1931, that they produce haemorrhage in tumours (150). This is discussed in detail in *Approaches to Tumour Chemotherapy* (151). The purified polysaccharide from *Serratia marcescens* was eventually tested on three patients with sarcomata and one with Hodgkin's disease in Boston (152) and nine patients in Philadelphia (153). Even the most potent preparations were pyrogenic and in clinical use caused the body temperature to rise to 108°F. This finding reminds one of the therapeutic success obtained by physical treatment in which tumours were heated above a certain critical temperature [cf. (154)].

Podophyllin.—Podophyllin obtained from the rhizome portion of *Podophyllum peltatum* is described as having an effect on cells like that of colchicine. It has been used in treatment of condylo-mata accuminata (155). When podophyllin is given to mice with the Crocker sarcoma 180 it causes necrosis and retardation of growth of the tumour (156). It appeared to have less effect on the growth of a transplanted mammary adenocarcinoma in C3H mice. The cytological effects of podophyllin include an increase of mitotic figures in metaphase and complete suppression of anaphase and telophase figures (157).

Antifolic acid compounds.—Certain preparations of folic acid have been shown to inhibit growth and cause regression of spontaneous breast cancer in mice (158). Tests on man showed that pteroyldiglutamic acid and pteroyltriglutamic acid were nontoxic but had very little effect on malignant disease (159). Aminopterin or 4-aminofolic acid is a much more potent antifolic acid compound than the other pterins (160), inhibiting the growth-promoting action of folic acid in chickens in very small amounts. This substance inhibits the growth of the Rous sarcoma in chickens (161) and has produced temporary remissions in acute lymphatic and myelogenous leucaemias of childhood (162). Aminopterin was given in doses of 0.5 to 1 mg. daily and this dose produced severe depletion and aplasia of bone marrow and stomatitis with ulceration. Atrophic changes of intestinal mucosa occurred in some cases. The high toxicity of 4-aminofolic acid makes it an extremely interesting substance and the field of antifolic acid compounds will undoubtedly be further studied in relation to cancer chemotherapy.

Reductions in white cell counts have also been obtained with

p-aminobenzoic acid in leucaemias (163, 164) and it is possible that in high concentration it might have an antifolic action. Other experiments (165) have found an increase in the white cell count in leucaemia patients treated with *p*-aminobenzoic acid although the drug has shown a leucopenic action (166) in patients suffering from rickettsial infections.

Stilbamidine.—Stilbamidine or 4,4'-diamidinostilbene had been found effective in treatment of Kala-azar (167), a disease in which there is an increase in the globulin content of the serum. For this reason the drug has been used in treatment of cases of multiple myeloma (168) in which condition the serum protein distribution is also abnormal. The drug was administered to patients on a diet low in animal protein and gave relief of pain in most cases without curing the condition. The effect on multiple myeloma may be connected with the fact that stilbamidine destroys neoplastic cells grown in tissue culture (169). The treatment appears to cause deposition of precipitates of ribose nucleic acid in cells (170).

Only a few of the fields in which progress in the chemotherapy of cancer has been made have been mentioned, but this may suffice to show that the advances are appreciable. Methods of assessment of therapeutic activity are improving and in many cases the lives of tumour-bearing animals can be prolonged by treatment. This prolongation can be used as the basis for assessing activity. The work is in a much more rational stage than it was and it is possible to advance partial explanations for some of the effects which are observed.

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METABOLISM OF PROTEINS AND AMINO ACIDS¹

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It is not proposed to cover the literature in the field indicated in the title with any degree of completeness. Instead a few topics have been selected, the discussion of which may be useful to other workers.

GENERAL

Whipple *et al.* (1) have continued their studies on the dynamics of protein metabolism. Ascites was produced by constriction of the inferior vena cava above the diaphragm. The periodic withdrawal of ascitic fluid drained the body protein reserves and this was equivalent to plasmaphoresis. A high protein and low sodium chloride diet reduced the loss of nitrogen in the ascitic fluid. Further experiments on the use of homologous plasma for maintenance of nitrogen equilibrium in protein-fasting dogs were also reported from the same laboratory (2, 3). A continued hyperproteinaemia with plasma levels of 10 per cent led to the excretion of large amounts of protein in the urine without any apparent kidney damage occurring. It is suggested that plasma proteins may pass readily through cell membranes and the glomeruli of the kidney. Dent & Schilling (4) suggest, on the basis of chromatographic analysis of ultrafiltrates of plasmas obtained from the jugular or portal vein, that the gut may absorb homologous plasma proteins as such, whilst heterologous proteins are first hydrolysed and then absorbed. Furthermore, direct evidence for this claim is desirable.

Essential amino acids.—The amino acid requirements of man (5) and the chick (6) have been reviewed. Womack & Rose (7) have reported their very careful experiments on the dietary significance of proline, hydroxyproline, and glutamic acid. The addition of either proline or glutamic acid to a diet devoid of the three amino acids and of arginine produced a significant increase in weight gains. However, supplementation with arginine was even more effective, in confirmation of earlier work (8). The best results were obtained by addition of all four amino acids to the basal diet.

¹ This review covers the period from November 1, 1947 to November 1, 1948.

The authors suggest that arginine, proline, and glutamic acid are converted into each other in the body and that the rates of the various synthetic processes are not sufficiently great for the requirements of optimum growth in the young animal. These experiments pose again the question as to the usefulness of the distinction between essential and nonessential amino acids (9) which suggests a clear-cut difference which does not in fact exist. It might be more useful to retain the term "essential" for those amino acids which, owing to the genetically determined synthetic disability of the organism, cannot be made at all or at a measurable rate out of the materials "ordinarily available" (8) in diets. A second group of "semiessential" amino-acids would comprise substances like cystine, tyrosine, arginine, etc., the dietary deficiency of which may become a limiting factor to growth under certain environmental conditions. The growth increments observed by Rose with diets not containing peptides are almost as high as any obtained with proteins. However, it has been shown in the same laboratory (10) that whole protein may still be superior to hydrolysed protein and this difference may be due to the presence of a growth factor of peptide nature, like strepogenin, in the former. On the other hand Frost & Sandy (11) found that free amino acids equal proteins in their nutritive value as measured by the regeneration of protein-depleted rats. In chicks also, there is no apparent difference in growth-promoting properties between amino acids and proteins (6). There is thus no definite evidence that strepogenin is of any importance for animal nutrition. Such differences as have been found may be due to other factors. Thus, it has been demonstrated that all amino acids required for protein synthesis must be present simultaneously in the right proportions (12). Geiger (13) has recently shown that, if supplementation of a diet deficient in lysine or tryptophane, or methionine is delayed, no growth occurs. The postabsorptive relative concentrations of amino acids in blood and tissues may, therefore, be slightly different in experiments in which proteins or amino acids are used.

Maintenance requirements.—There is a divergence of opinion as to maintenance requirements of the rat. Mitchell (14) believes that histidine, phenylalanine, and lysine are probably dispensable for the adult rat. However, Wissler *et al.* (15) observed that the absence of lysine, leucine, histidine, or phenylalanine from the diet invariably produced a negative nitrogen balance which was not

due to caloric deficiency, since forced feeding was used. It is of interest that with histidine a positive balance was observed for the first seven-day period. A similar result with respect to histidine has been reported for the dog by Elman *et al.* (16). Neuberger & Webster (17) also found that adult rats fed diets deficient in lysine lost weight and nitrogen as shown by carcass analysis when compared with paired-fed litter-mates. The phenylalanine requirement per day for adult man was found to be 1.9 gm. if given by mouth and 2.7 gm. if administered parenterally (18).

AMINO ACIDS IN BLOOD, TISSUES, AND URINE

Methods.—The ninhydrin method for the total α -amino acid nitrogen has been applied by van Slyke and others to blood and urine (19, 20, 21). The values thus obtained will include any peptide-like glutathione which has a free carboxyl next to a free amino group. For some amino acids fairly specific chemical micro-methods have been used; this applies to glycine (22), alanine (23), glutamic acid (24), and cystine (25). Glutamine has been determined by a variety of methods. Harris (26) used the liberation of ammonia on controlled hydrolysis, Hamilton (27) the decrease of α -amino acid nitrogen on heating, and Archibald (28) a glutaminase obtained from kidney which liberates ammonia; the results obtained by the three methods for plasma agree quite well. Krebs (29) has recently described a method which allows the estimation of both glutamic and glutamine; washed suspensions of *Clostridium welchii* decarboxylate glutamic acid (30) and deaminate the amide. A bacterial decarboxylase has also been used by Lawrie (31) to estimate tyrosine in urine.

Microbiological methods depending on stimulation of growth by amino acids with a suitable basal medium have been widely used in recent years. Although these methods give reasonably accurate results with hydrolysates of pure proteins, their application to blood, urine, or tissue extracts is more questionable. Interference may be caused by peptides, other conjugation products of amino acids, hydroxy or keto acids related to the substance to be estimated, and a variety of other compounds affecting growth. It has been demonstrated repeatedly that peptides can stimulate growth of a number of organisms used for assay purposes (32, 33, 34), and the same has been shown for α -keto and hydroxy acids (35). Moreover, Simmonds & Fruton (36) have recently reported

that a mutant strain of *Escherichia coli* requiring proline uses certain proline peptides more efficiently than proline itself. Dunn *et al.* (37), whilst using microbiological methods for amino acid analysis in blood and urine, have emphasized these difficulties. It is clear, therefore, that the figures obtained by microbiological assay may be subject to revision. Filter paper chromatography has also been used, especially by Dent (38); but the method is as yet not suitable for accurate quantitative estimation. Other factors important in the analysis of blood and tissue extracts, such as proteolysis and deproteinisation, have been stressed by Krebs (29) and by Christensen & Lynch (39).

Levels of amino acids in normal blood.—In normal man the α -amino acid nitrogen in plasma varies between 3.4 and 6.5 mg. per cent with a mean level of 4.5 mg. per cent and in cells is about 1.7 to 2.2 times as great (19, 21). Red cells contain relatively large quantities of glutathione, and this is most probably the main reason for the difference between cells and plasma. Erythrocytes are permeable to monoaminomonocarboxylic acids, although penetration is relatively slow, equilibration requiring about 24 hr. at 37°C. [Ussing (40)]. However, aspartic and glutamic acids diffuse only very slowly into the red cell. Christensen & Lynch (41) have only found small differences in amino acids other than glutathione, whereas Gutman & Alexander (42) report that the excess of amino nitrogen in the cells cannot be explained entirely by glutathione. Dunn *et al.* (43) using a microbiological method found a much higher tryptophane concentration in plasma than in cells. On ingestion of glycine the glycine content of plasma rose quickly, whilst the concentration in the cell changed only very slowly (44). It is likely that such differences between cells and plasma as have been found are due to the fact that the plasma concentrations change rapidly with feeding and with tissue metabolism, cells responding only slowly, with the result that complete equilibration is not obtained. Christensen & Lynch (39, 41) have also investigated the "bound" nonprotein amino nitrogen in blood; this fraction seems to be variable and to be generally very much smaller than the "free" amino acid fraction.

The glutamine content of human and dog plasma has been stated to vary between 6 and 11 mg. per cent (26, 27, 28); Roper & McIlwaine (45), using a different method, give a value of 7.2 mg. per cent for horse plasma. Harper (46), using a microbiological

technique, states that human plasma contains 6.4 to 11.6 mg. per cent glutamine and very little glutamic acid, whilst Prescott & Waelch (47), by a chemical method, find 0.6 to 1.7 mg. per cent before and 8 to 12.1 mg. per cent glutamic acid after hydrolysis. There is thus fairly good agreement and glutamine appears to represent about 18 to 25 per cent of the total α -amino acid fraction. Glycine and alanine in human plasma appear to average about 2 and 4 mg. per cent, respectively (42, 44), representing another 24 per cent of the amino acid nitrogen (48). Brown & Lewis (25) found values for cystine by the Sullivan reaction of 0.7 to 1.1 mg. per cent in human plasma filtrate. Values for the truly essential amino acids have all been determined by microbiological methods; these appear to lie between 1 to 3 mg. per cent (49) and to account, together with tyrosine and arginine, for 45 to 50 per cent of the amino nitrogen. Thus, the relative distribution of amino acids in plasma appears to differ from that found in proteins. Particularly noteworthy are the high glutamine, glycine, and alanine concentrations and the apparent absence of proline and aspartic acid (48).

Urine.—Urine again presents a different picture. Glutamine is only present in small amounts (28). Free glycine as shown by a chemical method averages about 120 mg. per 24 hr. (42), but a much larger amount of glycine up to 1 gm. per 24 hr. is present in a conjugated form, some of it extractable by ethyl acetate (44). This may explain the high values obtained by biological assay (37, 50). Microbiological estimations also indicate that in human urine histidine, cystine, and glycine predominate amongst the free amino acids (37, 50 to 53). The average daily excretions reported are: 150 to 250 mg. for histidine, 120 mg. for glycine, 60 to 110 mg. for cystine, and 50 to 60 mg. for threonine. Small amounts of other amino acids (10 to 30 mg. per day) are also excreted. Glutamic and aspartic acids are reported to be almost entirely present in a conjugated form and figures of 100 to 300 mg. per day for each have been observed. Lysine is also largely conjugated. In dog's urine lysine, histidine, and threonine are excreted in relatively large amounts (54). Free glycine, alanine, and other amino acids have also been observed on chromatograms (55). The concentrations of tryptophane and methionine in both urine and plasma appear to be low (43, 53, 56). Polarographic and colorimetric methods (57, 58) have given values of 40 to 80 mg. for the daily excretion of cystine by normal subjects. The daily output of tyrosine is stated

to vary between 11 and 37 mg. (31). The estimation of arginine by an enzymic method showed that urine from normal males had less than 15 mg. per l., whilst the plasma value was about 2.0 to 2.4 mg. per cent (59). The bromine reaction has been widely used for the estimation of histidine in urine [see, e.g., Chattaway (60)] and the results are of the same order of magnitude as those found by microbiological assay. Many other chemical methods have been used for the estimation of amino acids in urine, but most of the results are not very reliable.

Amino acids in tissues.—It was demonstrated a long time ago by Van Slyke (61) and confirmed by Luck (62) that tissues contain 5 to 10 times as much amino nitrogen as plasma. Hamilton (63), using the more specific ninhydrin method, found for various tissues of the dog values of α -amino acid nitrogen of 16 to 44 mg. per cent, i.e., about three to eight times higher than in plasma. Differences in the glutamine content were even more marked, a content of 225 mg. per cent for heart being found. The glutamine figures for skeletal muscle were 122, for brain 64, and for liver 45 mg. per cent. Christensen *et al.* (64, 65, 66) have extended these observations further. They found that in the guinea pig, though the absolute glycine concentrations in plasma and in tissues varied, the ratios for the extracellular and intracellular fluid were fairly constant and amounted to 33 for the liver and 8.3 for muscle. Glycine forms in the rabbit, but not in the rat, a very large proportion (up to 56 per cent) of the free amino acid fraction. Krebs (29) found that sheep's liver contains about 60 to 90 mg. per cent of glutamic acid and 20 mg. per cent of glutamine. The presence of peptides other than glutathione in various tissues is indicated by the data of Hamilton (27) and Christensen (64).

Effects of feeding amino acids.—Van Slyke & Meyer (61) in their classical experiments showed that intravenous injection of glycine and amino acid mixtures into dogs led to rise of blood and tissue amino nitrogen, being specially marked, but more transient in the liver than in muscle. Luck (62), using oral administration, showed that feeding of glycine, but not of alanine and other amino acids, increased specifically the amino nitrogen content of muscle. Recent work on man and other animals has shown that the increased α -amino nitrogen is not entirely due to the amino acid ingested; thus glycine produced a definite increase in the plasma alanine concentration (44). Even more striking are the results reported by

Christensen *et al.* (64) upon the mutual effects of changes in the tissue concentrations of amino acids. Thus, administration of L-proline (15 millimoles per kg.) to guinea pigs increased the plasma glycine level about seven times; the intracellular concentrations of glycine (liver and muscle) were increased to a much smaller extent. Similar, but somewhat less marked effects were observed with most other amino acids, i.e., changes in the ratio of intracellular to extracellular concentration of glycine on the administration of other amino acids and vice versa. Glutamic acid ingestion had a very different effect. Plasma amino acid levels were hardly affected, but the intracellular concentrations were increased. The detailed interpretation of these interesting observations is somewhat difficult, but the authors conclude that the various amino acids compete with each other for the mechanism by which cells concentrate amino acids and that glutamic acid has a somewhat specialized function in this process. This concentration mechanism is possibly connected with the synthesis of proteins and coupled with as yet unidentified energy-yielding oxidative reactions. Stern (67) has recently investigated such a reaction in guinea pig brain which contains normally 150 to 220 mg. per cent glutamic acid. On addition of glutamic acid and glucose to the medium *in vitro* the intracellular concentration of glutamic acid can be more than doubled. Malonate, oxide, and iodoacetate inhibit the accumulation. The problem of concentration of amino acids by bacterial cells has been studied extensively by Gale and his associates over the last few years [see review (163)]. Friedberg & Greenberg (68) have investigated the effects of the endocrine system on blood and tissue α -amino nitrogen. The same authors (69) have also examined the free amino acids in plasma and tissues of rats after feeding various amino acids. They have found in this species also that glycine is largely concentrated by muscle; glutamic acid was accumulated in the kidney, but apparently not in the liver. Hier (70) has studied the plasma levels of 12 amino acids of dogs at various intervals after giving one amino acid at a time by gavage. In almost all cases there was a marked and prolonged rise in the concentration of the amino acid fed, particularly so with histidine and threonine. Feeding phenylalanine increased the tyrosine level, but the reverse did not occur. Strangely enough, feeding of cystine or methionine did not affect the cystine level. This observation is in marked contrast to that of Brown & Lewis (71), who found in rabbits a considerable

rise in the plasma cystine level on either oral or parenteral administration of cystine.

Changes of amino acids due to other causes.—Bonsnes (72) has reported that the plasma α -amino acid nitrogen of pregnant women is decreased from the normal value of 4.2 to 4.3 mg. per cent to 3.0 to 3.2 mg. per cent. This rather surprising finding is largely explained by the investigations of Christensen & Streicher (65) which were carried out with guinea pigs and rabbits. In the guinea pig the foetal total plasma amino acid and glycine concentrations were five times greater than in the maternal plasma; the corresponding ratios for muscle were about three. Feeding an amino acid led to increases of amino acid levels in both mother and foetus; with glutamic acid the foetal plasma amino acid concentration rose from 10 to 40 mg. per cent. These results suggest that the placenta of the guinea pig can concentrate amino acids very efficiently. In the rabbit the plasma concentrations of foetus and mother are not so widely different, and the results suggest that the concentration mechanism in this species is largely located in the foetal tissues. The great avidity of placenta and foetal tissues for amino acids probably explains the decrease of α -amino acid nitrogen in human pregnancy. The association of growth with high intracellular amino acid levels is also demonstrated by another observation of Christensen *et al.* (66). It was found in rats that after partial hepatectomy, coincident with the most active phase of regeneration, there is a rise in intracellular amino acids and of glutathione and a fall in glutamine. Christensen & Lynch (73) also found that scorbutic guinea pigs show a marked reduction of glycine and glutamine in muscle and liver and a relative and absolute increase in residual amino acids.

RENAL EXCRETION OF AMINO ACIDS

A considerable number of data have been accumulated over the last few years on glomerular filtration and tubular reabsorption of amino acids, almost exclusively on dogs. The methods employed for the estimation of amino acids have either been the Van Slyke ninhydrin estimation or microbiological assay. Only rarely have specific chemical methods been employed (74). When the ninhydrin method was used, it was generally assumed that the rise of amino acid nitrogen observed was entirely due to the amino acid administered. As indicated above, this assumption may not be

quite correct. The objections against microbiological methods have already been fully discussed. In many investigations racemic compounds have been used and this may give misleading results with either method. If, as seems likely, the D-amino acid is less well reabsorbed, the urinary nitrogen will be largely composed of the D-amino acid and the plasma:urine ratio may be quite meaningless. Moreover, the D-compound may interfere with the reabsorption of the L-amino acid and this will affect the microbiological methods too. Some of the discrepancies of the results of various workers may be due to the use of the ninhydrin and microbiological methods respectively [Beyer *et al.* (75)].

However, in spite of these difficulties a fairly clear picture emerges. On the basis of the most widely accepted concept of the function of the glomerulus, amino acids must be present in the glomerular filtrate in the same concentrations as in plasma. But the loss of the amino acids in urine is very small and it follows therefore that reabsorption by the tubular cells must, under normal conditions, be almost complete. With glycine, it was found, both in man [Kirk (76)] and in the dog [Pitts (77)] that the amount which is reabsorbed is not constant, but rises steadily, as the blood level of the amino acid is increased. Thus, no definite renal threshold exists. Other simple amino acids behave in a similar manner and with most compounds studied blood levels could not be raised sufficiently high to reach T_m , the maximum value of tubular reabsorption; this applies to alanine [Pitts (78), Goettsch *et al.* (79)], valine, isoleucine, leucine, tryptophane [Beyer *et al.* (75)], phenylalanine, threonine [Russo *et al.* (80)] and methionine [Ferguson *et al.* (81) Wright *et al.* (82)]. Eaton *et al.* (83), using the ninhydrin method, found that the rate of reabsorption of valine, isoleucine, and leucine approaches a maximum in dogs, as the blood concentration is increased. Tyrosine and histidine are also almost completely reabsorbed in dogs [Doty (74), Wright *et al.* (82)]. The increase of the rate of tubular reabsorption with plasma level is explained by Pitts (77) as follows: The amino acid A is first combined in the tubular cell with a hypothetical substance B to give the compound AB which then breaks down to release A into the peritubular fluid. With low plasma concentrations the formation of AB is the rate-determining step and reabsorption increases with A; at higher concentrations of A the rate is determined by the decomposition of AB and thus becomes independent of the concen-

tration of A. Another possible explanation is that the T_m value is constant not for the whole kidney but only for individual tubules.

A few amino acids behave differently. Arginine and lysine are poorly reabsorbed, having T_m values in the dog of 13 and 11 mg. per min., respectively [Wright *et al.* (82), Pitts (78)]. This agrees well with earlier observations of Doty & Eaton, who found that intravenous administration of arginine (84) and lysine (85) leads to the excretion of the ingested amino acid in the urine, whilst with histidine which belongs to the first group, no such excretion was observed (86). Reabsorption with glutamic acid is less complete than with simple amino acids [Pitts (78), Loftspeich & Pitts (87)], but better than with arginine. It seems likely that the tubular cells of the dog are best adapted for the absorption of those amino acids which possess no net charge, such as monoaminomonocarboxylic acids. Histidine too, though it has a second basic group, exists largely as a neutral compound near pH 7.0. Lysine, arginine, and glutamic acid are ionised at that pH and are poorly reabsorbed. In this connection it is of interest that *N*-acetyltyrosine, in contrast to tyrosine, is almost completely excreted on intravenous administration [Doty (74)].

The reabsorption of glucose and glycine are independent of each other, whilst glycine and creatine appear to compete for the same mechanism [Pitts (77)]. A similar interference of creatine reabsorption is also shown by alanine and to a lesser extent by glutamic acid (78). Pitts suggests that nearly all amino acids may be reabsorbed by the same tubular mechanism and thus show mutual interference. However, the results of Beyer *et al.* (88) indicate that there are at least three, possibly more, groups of amino acids with regard to tubular reabsorption: (a) arginine, lysine, and histidine; (b) leucine and isoleucine; (c) glycine. The basic amino acids compete with each other, but not with leucine, isoleucine, and glycine; similar relationships were found for the other groups.

An interesting connection between tubular reabsorption, the capacity of an amino acid to contribute to urinary ammonia and the behaviour of amino acids *in vitro*, is revealed by Loftspeich & Pitts (87). Van Slyke *et al.* (89) had shown that glutamine is one of the precursors of urinary ammonia in acidotic dogs. The experiments of Loftspeich & Pitts demonstrate that certain amino acids which are known to be oxidatively deaminated by kidney enzymes such as glycine, alanine, and leucine also increase urinary ammonia; all these amino acids are easily reabsorbed. Arginine, ly-

sine, and glutamic acid are poorly reabsorbed, are not deaminated *in vitro*, and have no effect on ammonia production. It would be tempting, but probably premature, to conclude from this correlation that de- or transamination is involved in tubular reabsorption.

The capacity for reabsorption is most probably determined by genetic factors and may vary from species to species. This is well demonstrated by a recent paper of Friedman & Byers (90), who have shown that the presence of the large quantities of uric acid in the urine of the Dalmatian dog is mainly due to its inability to reabsorb uric acid from the glomerular filtrate. It is, therefore, not permissible to assume that tubular reabsorption in man resembles that of the dog. In fact, the results of Sheffner *et al.* (53) suggest that in man, histidine has a lower *Tm* value than lysine and this agrees with the relative distribution of the two amino acids in normal human urine.

Peptides obtained by enzymic hydrolysis of proteins appear to be excreted in the urine to a much greater extent than free amino acids. Thus Christensen *et al.* (91) observed that on intravenous infusion of a commercial casein digest into human subjects, about 50 per cent of the peptides were excreted, whilst the amino acids were well utilized. The elevation of the peptide level in the plasma was also more persistent than that of the amino acid fraction. On the other hand, a hydrolysate of fibrin containing peptides of only slightly shorter average chain length was somewhat better utilized (92). It is not clear whether this increased urinary excretion of peptides is due to a lower rate of tubular reabsorption or to slower uptake by tissues.

AMINO ACID EXCRETION UNDER PATHOLOGICAL CONDITIONS

Mice fed diets the protein component of which is deficient in an essential amino acid excrete, as observed by Pearce *et al.* (93), increased quantities of other amino acids in the urine. The same authors [Saubert *et al.* (94)] also showed that there is a rough inverse relationship between the biological value of the protein fed and the degree of amino aciduria (94). With rats this phenomenon was somewhat less marked. It is to be expected that animals cannot use other amino acids in the diet, if one of the essential amino acids is missing. But the fact that a large proportion of these amino acids is not oxidized, but excreted, is surprising and is explained by the authors as being due to an inhibition of the oxidative breakdown in the tissues.

Considerable interest is attached to recent investigations on the excretion of increased amounts of amino acids in certain familial diseases. The presence of the diamines, cadaverine and putrescine, which are derived from lysine and ornithine respectively, in the urine of a cystinuric was reported by Udranzky & Baumann (95) more than 60 years ago. Later Ackermann & Kutscher (96) and Hoppe-Seyler (97) isolated crystalline lysine derivatives from cystinuric urines; the latter obtained a yield equivalent to 0.1 gm. of lysine per l. of urine which, even without allowing for losses during preparation, is many times the quantity present in normal urine. Yeh *et al.* (50) have recently investigated the amino acid excretion in the urine of a cystinuric by microbiological methods. They found a considerable increase in cystine, arginine, and lysine and a corresponding decrease in glycine and histidine. The total amino acid output was not appreciably increased. No blood levels of amino acids were determined.

A condition which is possibly related is the Fanconi syndrome, which is characterized by rickets due to poor tubular reabsorption of phosphate, glycosuria also of renal type, and the deposition of cystine within the reticuloendothelial system (cystinosis) in some cases. McCune *et al.* (98) demonstrated in one case the presence of greatly increased amounts of amino acids in the urine, whilst the blood nonprotein nitrogen was within normal limits. The authors conclude that the amino aciduria was due to partial failure of tubular reabsorption. Dent (55) has carried out more extensive chemical investigations on three other cases. He found in two cases a moderate increase and in the third a very great increase of amino acid nitrogen in the urine, whilst the plasma amino acid nitrogen was normal. Dent also identified various amino acids chromatographically and concluded that serine, threonine, leucine, and valine were most prominent. No actual figures for individual amino acids were obtained. The data are consistent with the explanation that amino acids, like phosphate and glucose, are excreted, because they are incompletely reabsorbed by the tubules. No simple explanation can be given on this basis for the cystinosis. The fact that cystinuria has been observed in some of the patients or their relatives suggests that this inborn error of metabolism might also be renal in nature.

Another familial disease in which increased amino acid urinary excretion has been observed is hepatolenticular degeneration (Wilson's disease). Uzman & Denny-Brown (99) have found amino

nitrogen values in the urine of 800 to 1,500 mg. per day. About 10 to 12 amino acids were identified chromatographically, and alanine, aspartic acid, and glutamic acid were actually isolated. The blood amino nitrogen values were definitely raised being 5.5 to 6.3 mg. per cent. Eckhardt *et al.* (100) have obtained similar results with five other cases; in addition they determined the ten "essential" amino acids microbiologically and the figures indicate that the relative proportions are not strikingly different from those found in normals. The slightly raised blood amino acid levels found by both groups of workers, the failure of intravenous infusion of amino acids to increase greatly excretion (Eckhardt *et al.*), and the absence of any other signs of failure of renal absorption raise doubts as to whether the observed increased excretion of amino acids is tubular. In many cases of progressive muscular dystrophy an increased amino acid excretion has been observed by Ames & Risley (101).

Renal factors also play a part in other inborn errors of metabolism. In phenylketonuria, large amounts of phenylpyruvic acid are excreted in the urine; the phenylalanine content appears, according to the observations of Dann *et al.* (102), to be only slightly raised. The blood of the phenylketonuric contains greatly increased amounts of phenylalanine [Fölling *et al.* (103), Jervis *et al.* (104)] but no phenylpyruvic acid. This difference between blood and urine is almost certainly explained by the effect of the kidney. The phenylketonuric is unable to convert phenylalanine to tyrosine [Jervis (105)] and the former, therefore, accumulates in blood and tissues and is filtered by the glomeruli. However, since the reabsorptive capacity of the tubules of the phenylketonuric is probably normal, almost all the amino acid is reabsorbed. However, a certain proportion is oxidatively deaminated to the keto acid which is either not at all or only incompletely reabsorbed and is therefore excreted in the urine.

In alcaptonuria the blood level of homogentisic acid is very low, as found by Neuberger *et al.* (106), who considered the possibility that the inborn error might be due to a failure of reabsorption. However, Leaf & Neuberger (107) showed recently that the kidney threshold for this acid is exceedingly small even in normal subjects, and it appears, therefore, more likely that alcaptonuria is due to a metabolic lesion. However, the very low blood concentration coincident with a high urine concentration suggest that homogentisic acid is formed in the kidney.

INTERMEDIARY METABOLISM OF SOME AMINO ACIDS

Glycine.—Shemin & Rittenberg showed in two fundamental papers (108, 109) that (a) glycine or a substance derived specifically from glycine is the precursor of the nitrogen of the protoporphyrin of the haemoglobin of man and the rat; (b) that the porphyrin inside the erythrocyte is metabolically stable in normal subjects until the cell dies; and (c) that the average life span of the red cell in man is about 127 days. It has now been reported from the same laboratory (110) that porphyrin formation can be demonstrated *in vitro* with blood obtained from patients suffering from sickle cell anaemia. The same authors (111) have also shown that such a synthesis *in vitro* occurs with nucleated avian red cells. Muir & Neuberger (112) have oxidized mesoporphyrin obtained from rabbits fed isotopic glycine and found identical isotope concentrations in acidic and neutral oxidation products, indicating that glycine almost certainly provides the nitrogen atoms of all four pyrrole rings. Identical results have been obtained with avian cells *in vitro* by the Columbia group using a different method of degradation (164).²

Altman *et al.* (113) have shown that the α -carbon atom of glycine is incorporated into porphyrin; by contrast the carboxyl carbon is used, as found by Grinstein *et al.* (114), for the synthesis of the globin, but not for that of the haeme moiety of the haemoglobin molecule. The possibility that glycine might first be converted to serine and ethanolamine was examined by Muir & Neuberger (112), who fed N^{15} containing ethanolamine to rats. However the N^{15} content of the porphyrin was much lower than after feeding an equivalent amount of isotopic glycine.

Glycine is also the precursor of at least part of the uric acid molecule. Buchanan *et al.* (115) using C^{13} showed that in the pigeon the carboxyl group of glycine provides carbon atom 4 of uric acid. Evidence was also produced that the nitrogen in position 7 is also derived from glycine, and it is suggested that the methylene group of glycine is the precursor of carbon 5. Shemin & Rittenberg (116) have shown that in man the nitrogen of glycine is the source of the nitrogen 7 of uric acid. Abrams *et al.* (117) degraded guanine derived from the nucleotide fraction of a yeast grown in a medium containing nitrogen labelled glycine. They found a high

² The results of the American workers have now been published in full [WITTENBERG, J., AND SHEMIN, D., *J. Biol. Chem.*, **178**, 47-51 (1949)].

isotope content for nitrogen derived from position 7, thus suggesting that glycine functions in a similar manner in the biosynthesis of purines in yeast and in the formation of uric acid in mammals and birds.

Greenberg & Winnick (118) studied the metabolism in the rat of glycine labelled with C^{14} in the methylene group. Most of the activity of the proteins was due to glycine, but high specific activities were also observed in glutamic and aspartic acids and in arginine. No activity was found in serine. The latter observation is surprising in view of the findings of Winnick *et al.* (119), who incubated liver homogenate with either C_1 or C_2 labelled glycine. Under these conditions most of the activity of the protein was found to be due to serine, and it would thus appear that the conversion of serine to glycine discovered by Shemin (120) is reversible, at least *in vitro*.

Lysine.—Borsook *et al.* (121) have studied the metabolism of lysine labelled with C^{14} in the ϵ -position with guinea pig liver homogenates. Evidence was obtained that lysine is converted though, rather slowly, to α -aminoadipic acid. Dubnoff & Borsook (122) showed that the latter, like lysine, can aminate citrulline to arginine in kidney slices. The relative rates of the two reactions suggest, but do not prove that, in this tissue too, lysine is metabolized by oxidation in the ϵ -position.

In *Neurospora*, evidence has been obtained by Mitchell & Houlahan (123) that α -aminoadipic acid is a precursor of lysine. One of four mutants requiring lysine for growth can utilize D or L- α -aminoadipic acid instead of lysine. The conversion of a carboxyl to an amino group is a most unexpected biological reaction and the authors suggest that an amide may be the intermediate. Complicated and not yet fully explained relationships between arginine, lysine, and pyrimidines are indicated in this and another paper [Houlahan & Mitchell (124)].

Another aspect of lysine metabolism has recently been investigated by Gaudry and his associates. ϵ -Hydroxy- α -aminocaproic acid was prepared (125) and shown to be unavailable for growth to rats fed a lysine-deficient diet (126). Moreover, the hydroxy compound was found to produce a marked anaemia (127). This observation explained the older findings of Hogan *et al.* (128), who had already obtained evidence that the anaemia produced by feeding deaminized casein to rats was associated with a derivative of lysine.

Sulphur-containing amino acids.—The conversion of methionine to cystine in the rat has been further studied by du Vigneaud and his associates. Anslow *et al.* (129) prepared the four possible optically active isomers of cystathionine and investigated their effect on growth. The nomenclature used requires some explanation. L-Cystathionine refers to the isomer containing the natural or L-configuration for the cysteine-like and homocysteine-like moieties, whilst allocystathionine is used for the isomers in which one of the two asymmetric carbon atoms has the unnatural or D-configuration. In the latter cases the L-prefix refers to the isomer having the L-configuration in the cysteine-like portion of the molecule. The D-isomers refer to the optical isomers of both L-cystathionine and L-allocystathionine respectively. D-Cystathionine and D-allocystathionine did not support growth at all, whilst L-allocystathionine, like L-cystathionine, supported growth on a diet free of cystine and low in methionine. But unlike the latter, the allo compound also appeared to replace homocysteine, and the authors suggest that whilst L-cystathionine is cleaved to cysteine, L-allocystathionine yields D-homocysteine which is known to be available for growth. This inference was fully substantiated by Anslow & du Vigneaud (130), who found that liver extract hydrolyzed L-allocystathionine to D-homocysteine and the D-allo compound to D-cysteine. Since the latter is not available for growth instead of L-cystine, the negative feeding experiments are fully explained. The point of cleavage appears to be determined by the configurations of the two asymmetric centres. If they are both L, cysteine is formed. If one of them is D, the one C-S linkage nearest to the asymmetric carbon atom of L-configuration is split. If both centres have D-configuration cleavage is very slow. While in mammals the conversion of L-methionine to L-cystine through L-cystathionine is not reversible, the chain of reactions is reversed in *Neurospora* and *E. coli*. Horowitz (131) investigated the nutritional requirements of four single gene mutants of *Neurospora* which were unable to synthesize methionine. The results obtained can most simply be explained by assuming that *Neurospora* normally synthesizes methionine from cysteine through cystathionine. This idea was fully confirmed by the fact that one of the mutants accumulated cystathionine which was isolated and fully characterized. In a second paper Teas, Horowitz & Fling (132) showed that in *Neurospora* the four carbon fragment of cystathionine originates from γ -hydroxy- α -aminobutyric acid (homoserine). Most surpris-

ing is the finding that the latter is also apparently a precursor of threonine. Lampen *et al.* (133) on the basis of experiments on mutant strains of *E. coli* suggested that the conversion of methionine to cystine in this organism might be reversible. A more detailed analysis was carried out by Simmonds (134) on two strains of *E. coli* K-12. One strain required methionine; the latter could be replaced by D-methionine, L-cystathionine, or D-allo-cystathionine, but not by the other two isomers. The fact that cystathionine stimulates growth suggests that the latter is converted to methionine; the stereochemical specificity indicates that only the two isomers of cystathionine which can yield L-homocysteine can be utilized for methionine synthesis. It appears, therefore, that the stereochemical specificities of the enzyme of the rat and of *E. coli* are different.

Two other thio ethers have recently been examined for their growth effects. Jones *et al.* (135) have found that L-lanthionine [S-bis-(β -amino- β -carboxyethyl)-sulphide], in contrast to the meso compound, can replace cystine in the diet of the rat. This suggests that the body can split the symmetrical thio ether lanthionine to yield L-cystine, if both asymmetric centres have L-configuration. The next higher symmetrical homologue of lanthionine homolanthionine was prepared by Stekol (136) as a mixture of inactive and meso forms. This compound was found by Stekol & Weiss (137) to replace cystine, but not methionine in the diet of the rat. The authors suggest that this rather surprising finding might be explained by assuming that the thio ether condenses with serine to form a sulphonium base which is then transformed to cystathionine.

As might be expected γ -hydroxy- α -aminobutyric acid does not replace methionine or homocystine in the diet of the rat (138). The corresponding methoxy compound (methoxinine) which is an oxygen analogue of methionine is, as observed by Shaffer & Critchfield (139), quite toxic, but has some lipotropic activity. Another, rather striking example, of stereochemical specificity has recently been observed by Wilson & du Vigneaud (140), who found that L-penicillamine (β , β -dimethylcysteine) is toxic to rats. This toxicity is counteracted by choline and ethanolamine. The D isomer which is a hydrolytic product of penicillin is not toxic.

Tyrosine.—It has been known for some time that tyrosine increases the excretion of ketone bodies. But it has only recently been demonstrated by labelling with C¹⁴ that the β -carbon atom of

the side chain is actually converted to acetoacetate. Winnick *et al.* (141) using rats have found that most of the radio activity of the tissue proteins was due to tyrosine itself, but there was a fairly high activity in the urinary ketone fraction. Weinhouse & Millington (142) working with liver slices showed that the β -carbon atom of tyrosine appears almost entirely as the α -carbon atom of acetoacetate. They suggest that the carboxyl carbon of the keto acid is derived from the α -carbon of tyrosine, whilst two of the ring carbon atoms supply the β - and γ -carbons of acetoacetate.

The role of ascorbic acid in the metabolism of tyrosine is still quite obscure. Painter & Zilva (143) found that the excretion of *p*-hydroxyphenylpyruvic acid and related compounds by guinea pigs deprived of ascorbic acid begins almost as soon as the vitamin is removed from the diet, i.e., at a time when the tissues still contain large amounts of ascorbic acid. Levene *et al.* (144) extending their work on tyrosine metabolism from premature to full-term infants found that with a diet high in protein and deficient in ascorbic acid, administration of additional tyrosine and phenylalanine led to excretion of keto acids and of tyrosine. Ascorbic acid did not prevent the excretion of these metabolites when large doses of the two amino acids were given. The excretion of aromatic keto acids by vitamin C-deficient guinea pigs can also be reduced by the administration of liver preparations with antipernicious anaemia activity (145), and similar effects have been reported for pteroylglutamic acid (146).

Fishberg (147) has identified the reducing substance excreted by patients suffering from enterogenous cyanosis as *p*-quinone acetic acid, the quinone of homogentisic acid. It would be of great interest to investigate the connection which may exist between the disturbance of the metabolism of aromatic amino acids and met-haemoglobinanaemia, which is the dominant feature of this condition. Gibson (148) has recently advanced good reasons for the belief that the red cells of patients suffering from the familial forms of this disease are deficient in the coenzyme factor I.

Neuberger (149) has synthesized and resolved 2,5-dihydroxyphenylalanine and discussed the possible intermediate steps involved in the conversion of tyrosine to homogentisic acid. Although there is very strong evidence that this reaction represents quantitatively the most important pathway of tyrosine metabolism, the exact mechanism of the transformation is still obscure. The oxidation of tyrosine or its keto acid involves an attack by an

electrophilic reagent, and the three positions in the ring with the highest electron densities are the two ortho and the para positions. Preferential attack in the para position might be facilitated by suitable substitution in the side chain (such as exists in the enolic form of the keto acid) or by steric factors in the enzyme. The most reasonable picture of such an oxidation appears to be the removal of two electrons from one of the resonating structures of the phenoxide ion leading to a carbonium ion. The latter would combine with an hydroxide ion and thus give a hydroxydienone which would readily rearrange itself to a substituted *p*-quinol. Similar reactions in unrelated fields have recently been described (150, 151, 152) and are most probably analogous to migrations observed in pinacol and similar rearrangements.

The conversion of tyrosine to homogentisic acid is somewhat similar to that of diiodotyrosine to thyroxine. Johnson & Tewkesbury (153) and Harington (154), who have discussed the mechanism involved, assume that the first step is an oxidation in para position to the existing hydroxyl group. The preferential attack in the para position in this case requires, of course, no explanation. The above authors also assume that only one electron is removed leading to a free radical which, according to Harington, combines with another free radical having the odd electron on the oxygen, thus giving an ether one half of which has a dienone structure. It is, of course, possible to formulate the first step as the removal of two electrons as discussed above for tyrosine. Ether formation in the case of tyrosine is less likely, since the concentration of phenoxide ions is smaller than with diiodotyrosine which has a much more acidic phenolic group. The rearrangement of the dienone structure of the thyroxine intermediate is associated not with the migration, but with the elimination of the side chain. An interesting parallel for such a fission has recently been observed by Woodward & Witkop (155) with ketoyobirine. These authors assume that the driving force of the reaction is the tendency of the dihydropyridine ring to become aromatic and this explanation also applies to the dienone. The actual fate of the side chain is still obscure. Johnson & Tewkesbury (153) have reported that they had obtained qualitative evidence for the presence of pyruvic acid. However Pitt-Rivers (156) has investigated the reaction with the *N*-acetyl derivative which gave greatly increased yields of thyroxine and reported that the solution after incubation and hydrolysis contained small amounts of alanine as observed on filter paper chromato-

grams. As the author herself points out, the significance of this finding is doubtful and the identification of the eliminated fragment is still outstanding.

The other type of oxidation of tyrosine, i.e., in ortho position leads through 3,4-dihydroxyphenylalanine to epinephrine. This has been proved with the aid of isotopes by Gurin & Delluva (157), who recovered radioactive epinephrine after administration of phenylalanine labelled with either tritium or C^{14} . Melanin is generally assumed to be derived from 3,4-dopa, and the mechanism proposed by Raper which assumes the intermediate formation of 5,6-dihydroxyindole have recently been confirmed by Mason (158). The synthesis of 5,6-dihydroxyindole has been announced by two groups of workers (159, 160). However, the structure of melanin itself is not yet settled, but Harley-Mason (161) has shown that 5,6,5',6'-tetrahydroxyindigo, which he synthesized, differs markedly from melanin and a different structure has, therefore, to be considered [see also Burton (162)]. It appears possible that some melanins may be derived not from 3, 4-, but from 2,5-dopa.

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INTERMEDIARY METABOLISM OF PHOSPHORUS COMPOUNDS¹

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SYNTHETIC UTILIZATION OF PHOSPHATE BOND ENERGY

Although it had become rather clear that energy delivery from fermentative, as well as from oxidative, metabolism is geared through the generation of energy-rich phosphate bonds, there had existed until recently a vacuum on the receiving side. For a long time only relatively few instances of phosphate bond utilization were recognizable. During the last few years, however, the number of synthetic reactions driven by an influx of energy-rich phosphate bonds has increased impressively. Peptide formation (19, 43, 58, 170), the synthesis of urea (40, 158), transmethylation from methionine (25), the synthesis of α - and β -keto acids (93, 169), of fatty acids (11), and the process of bioluminescence (131), to name the most important advances, appear now to belong into this group.

In most cases as yet a general dependence of such condensations on the availability of adenosinetriphosphate (ATP) was recognized and little information has been gained about the nature of phosphorylated intermediaries expected to be formed. A promising, although still preliminary, advance has been made, however, towards a final characterization of the phosphorylated acetyl precursor (93), the intermediary in acetylation, in keto and fatty acid and, most likely, in citric acid synthesis (91).

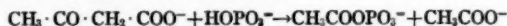
CONDENSATIONS INVOLVING ACETATE ACTIVATION

Acetoacetate splitting and synthesis.—After the discovery of the phosphoroclastic splitting of pyruvate (101, 187), an analogous reaction with acetoacetate appeared likely. Such phosphoroclastic splitting of acetoacetate to acetylphosphate and acetate has now been demonstrated with cell-free preparations of *Clostridium kluyverii* by Stadtman & Barker (174). A disappearance of inor-

¹ This review covers the period from December, 1946 to December, 1948.

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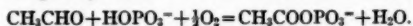
ganic phosphate accompanied the reaction and was related to equivalent formation of acetylphosphate and free acetate. The fractionation (123) and hydroxamic acid (122) methods were used with identical results for the determination of acetylphosphate and the reaction is formulated as follows:



The reverse reaction, condensation of acetate plus ATP, to acetoacetate was found by Soodak & Lipmann (169) to occur in acetone pigeon liver extracts. A milliliter of extract formed three to four micromoles of acetoacetate. The reaction is dependent on the presence of coenzyme A and thus is essentially an acetylation of acetate. The splitting of acetoacetate (169a) in pigeon liver extracts appears to be a rather complicated reaction, not just of the phosphoroclastic type but rather requiring ATP. Indications for an unstable intermediary were found. Acetoacetate may almost quantitatively serve as acetyl donor for sulfanilamide but a formation of free acetate could not be detected without acceptor.

Previously the change of free energy with hydrolysis of acetoacetate (117) has been calculated to be $-16,000$ calories, a ΔF_0 approximately equal to that for acetyl phosphate hydrolysis. Like the analogous reaction with pyruvate, the phosphoroclastic split of acetoacetate, therefore, should occur with little change of free energy which appears now to be in good agreement with experimental data.

With the same preparation of *C. kluyverii* Stadtman & Barker showed (174), furthermore, that with the oxidation of acetaldehyde, an equivalent of phosphate was converted to acetyl phosphate:



Of particular significance is the observation of a synthesis in their extract of butyric and caproic acid (11) from synthetic acetylphosphate, with hydrogen as the reductant. The unusual activity of synthetic acetylphosphate possibly as a direct acetyl donor is noteworthy. It is of further interest that in the same system acetoacetate could not be reduced to butyric acid.

Pyruvate synthesis.—Utter, Lipmann & Werkman (187) had found previously an incorporation into pyruvate of isotopic acetate with *Escherichia coli* extract when ATP was added. Kaplan & Lipmann (93) verified the isotope experiment by demonstrating a net synthesis of pyruvate from acetate plus ATP and formate in

dialyzed extracts of *E. coli*. Recently, Strecker, Krampitz & Wood (176) prepared isotopic acetylphosphate. They found that the synthetic acetylphosphate did not yield significant amounts of acetyl in pyruvate synthesis.

The acetyl precursor.—It had been variously observed that acetate plus ATP will furnish an acetyl precursor in enzymatic reactions where synthetic acetylphosphate was found inactive (115). Nevertheless, an apparent reverse formation of acetylphosphate from ATP plus acetate had been observed with bacterial extracts (120). This reaction between ATP and acetate was recently studied in greater detail with *E. coli* extracts by Kaplan & Lipmann (93, 94). Increasing yields of a compound of the analytical behavior of acetylphosphate were obtained by increasing the acetate concentration up to 0.4 molar. The use of phosphopyruvate as a phosphate donor with carrier amounts of adenosinediphosphate (ADP) proved furthermore advantageous. This showed definitely that no part of ATP was carried into the acetyl derivative. It was found also that together with ADP and additional acetate, the synthetic acetylphosphate may serve as a phosphate donor to form the new compound. This explains the earlier observation of pyruvate synthesis with synthetic acetylphosphate in crude extracts of *E. coli* (120).

These recent experiments indicate that the reaction between ATP and acetate is not a true reversal of the reaction between synthetic acetylphosphate and ADP. The compound formed is in many respects different from synthetic acetylphosphate. Most important is its activity as acetyl donor in several enzymatic systems. It was found active in the pyruvate synthesis with formate in dialyzed *E. coli* extract (93) and with acetylation of sulfanilamide in pigeon liver extract (95). The compound is furthermore distinguished by its relative resistance to acetylphosphatase (93) prepared from skeletal muscle. After short incubation with 0.1 *N* HCl at room temperature however, the compound becomes indistinguishable from synthetic acetylphosphate and loses its enzymatic activity. A preliminary purification of the barium salt by fractionation with alcohol was reported (93).

Wilson, Krampitz & Werkman (196) have demonstrated the reversal of the phosphoroclastic split of pyruvate in *Clostridium butylicum* suspected earlier by Lipmann & Tuttle (121). Tagged carbon dioxide appears in the carboxyl group of pyruvate in a

preparation which is dissimilating pyruvate to carbon dioxide, hydrogen, and acetylphosphate. Addition of hydrogen not only decreases the amount of pyruvate split (cf. 196) but also increases the amount of carbon dioxide fixed. Carboxyl labelled acetate is incorporated into pyruvate significantly only when ATP is added. Synthetic monoacetylphosphate in combination with adenylic acid is more effective than ATP in converting the isotopic acetate into pyruvate acting probably as a phosphate donor (cf. 93).

The reversibility of the dismutation, $2 \text{ pyruvate} + \text{phosphate} \rightleftharpoons \text{lactate} + \text{acetylphosphate} + \text{CO}_2$, in *Staphylococcus aureus* was shown by Wikén *et al.* (195). In a cell-free preparation isotopic carbon dioxide was fixed in the carboxyl group of pyruvate but only when ATP was added. A carbon dioxide fixation by a reversed pyruvate dismutation had not been observed so far; it is particularly noteworthy because such a dismutation of pyruvate occurs in animal tissues.

Ketoglutarate synthesis.—A new instance of reductive α -carboxylation, the long expected carboxylation of succinate (123) to ketoglutarate is now reported by Ajl & Werkman (1). Using isotopic carbon dioxide a reversal of the decarboxylation of ketoglutarate to carbon dioxide and succinate in *E. coli* was observed. The fixation of carbon dioxide in ketoglutarate appears to be promoted by addition of ATP, and intermediate formation of a succinyl phosphate is indicated.

ACETYLATION STUDIES: SYNTHESIS OF PEPTIDE BONDS

The acetylation of aromatic amines is essentially a peptide bond formation; it is now heading the list of a series of analogous reactions. The participation of coenzyme A (119), a pantothenic acid derivative, particularly prompted an intensive study of the process of acetylation.

A partially purified enzyme was prepared by Kaplan & Lipmann (92) and the following components were found necessary: ATP, acetate, coenzyme A, and cysteine. The reaction between ATP and acetate in pigeon liver extracts, shown by trapping acyl phosphate with hydroxylamine (121), was restudied (95) and also found to require coenzyme A. This suggested that although synthetic acetylphosphate was inactive, some acyl phosphate compound should be involved as an intermediate in the acetylation

reaction. As such, the reaction product of ATP with acetate in *E. coli* extracts was now found to be active (95). As with sulfanilamide acetylation, coenzyme A was found to be essential for choline acetylation [Lipmann & Kaplan (118)]. Coenzyme A is identical with the heat-stable factor which was independently reported by Feldberg & Mann (62) and by Nachmansohn & Berman (142). Hydroxylamine intercepts the acetylation of choline; in its presence acethydroxamic acid results from the reaction between ATP and acetate in extracts of pigeon brain acetone powder (95).

It has been suggested by Lipton & Barron (124) that citrate is more active than acetate as a precursor of the acetyl groups in acetylcholine. Evidence by Kaplan & Lipmann (92) and by Nachmansohn & Weiss (143) indicates that citrate is active only because of its repressive effect on the adenosinetriphosphatase action by removal of divalent ions. In isolated systems where there is little adenosinetriphosphatase activity, citrate has no effect on the acetylation reaction. It appears from these enzyme studies that acetate is the sole direct precursor of acetyl groups in acetone powder extracts for the acetylation of sulfanilamide and of choline.

Glutamine synthesis.—The mechanism of synthesis of the amide bond appears to be quite similar to that of sulfanilamide acetylation. Speck (170, 171), using pigeon acetone liver extract such as used for sulfanilamide acetylation, found an anaerobic synthesis with ATP as the condensing agent. Glutamine was formed from glutamate and ammonia, and glutam-hydroxamic acid with hydroxylamine as acceptor instead of ammonia. Elliott (58, 59) independently obtained the same reactions in acetone-dried sheep brain extract. No acyl phosphate was found to accumulate in the absence of acyl acceptor. In the presence of ammonia or hydroxylamine the phosphate liberated from ATP was practically equivalent to glutamate or hydroxamate formed. Cysteine and magnesium were necessary for this synthesis. The close resemblance between sulfanilamide acetylation and glutamine synthesis is evident. In both cases the primary reaction appears to be an activation of the carboxyl group by a reaction with ATP. In both cases the physiological acceptor may be replaced by hydroxylamine. As yet, however, there is no indication that coenzyme A is involved in glutamine formation. Elliott & Gale (60) also extracted

from *S. aureus* an enzyme which forms hydroxamic acid on addition of ATP and glutamate. This system is competitively inhibited by methionine oxide.

Hippuric acid synthesis.—The understanding of the mechanism of this much-studied reaction has been advanced considerably. It appears to be essentially analogous to the two above-reviewed peptide bond formations.

Borsook & Dubnoff (23, 24) obtained formation of hippuric acid in guinea pig liver homogenates from benzoate and glycine. Aerobic conditions were necessary; the addition of ketoglutarate and adenylic acid doubled the yield of hippuric acid.

Cohen & McGilvery (41, 43) made an intensive study of *p*-aminohippuric acid synthesis in rat liver and kidney slices and in rat liver homogenates. ATP but not adenylic acid supports a synthesis anaerobically in liver homogenates (42). N-phosphoglycine was inactive in the reaction. The evidence points to the activation of the carboxyl group in benzoic acid as being the primary step in hippuric acid formation. In contrast to acetylation and glutamination, the hippuric acid synthesis is not found in the tissue extract but in the insoluble residue.

Glutathione synthesis.—This synthesis may be considered the most representative example of an ATP-promoted peptide synthesis. Bloch & Anker (20) demonstrated aerobic incorporation of labelled glycine into glutathione in rat liver slices and also in homogenates (19) although the absolute amount of glutathione decreased. With ATP added, this synthesis was found to proceed anaerobically to the same extent as aerobically. N-labelled acetylglycine which in slices is rapidly incorporated into glutathione was inactive in homogenates. This may rule out the possibility of acetylglycine being an intermediate in this peptide bond formation (cf. 22).

Protein synthesis.—As yet no direct evidence of the activity of ATP in protein synthesis has been reported, although some circumstantial evidence suggests that phosphate bond energy may be involved. The need of oxygen for the synthesis of labelled proteins from radioactive amino acids in tissue slices and homogenates was shown by Zamecnik *et al.* (198). An observation of Frantz *et al.* (63) appears significant. 2,4-Dinitrophenol, which uncouples phosphorylation from oxidation (126), inhibits in slices of both normal and carcinomatous rat liver the incorporation of alanine

into protein without inhibiting or rather with increasing respiration. The suggestion emerges that phosphate bond energy should be involved in the incorporation of alanine into proteins. A similar suggestion appears from Hotchkiss' work with staphylococci (82, 83). Here, likewise, 2,4-dinitrophenol inhibits nitrogen and phosphate assimilation without preventing respiration. Inhibitor studies by Spiegelman *et al.* (172) also furnish valuable circumstantial evidence for the relation of synthesis of cell constituents to an availability of phosphate bond energy.

Chemical studies with acyl phosphates.—Bentley (12) published a series of significant observations on the mechanism of monoacetylphosphate hydrolysis. Using heavy oxygen-containing water as indicator, he found that an acetyl split occurs between carbon and oxygen at alkaline reaction. At neutral or acid reaction, i.e., in the physiological region, however, a phosphoryl split occurs between oxygen and phosphorus. The acetylphosphate used in these experiments was synthesized by a new and elegant procedure through the action of ketene on phosphoric acid (13).

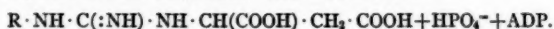
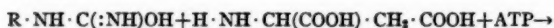
Chantrenne (32, 33) looking for an explanation of the apparent insufficiency of synthetic monoacylphosphate to serve as acyl donor, did some interesting model experiments on hippuric acid synthesis. He prepared dibenzoyl phosphate which in contrast to the mono compound was found to react nonenzymatically with glycine to form hippuric acid. Likewise, the phenyl benzoyl phosphate acted as a good benzoyl donor (33). He draws the conclusion that a second substituent on phosphoric acid facilitates the acyl transfer function. In confirmation it was found (95) that diacetylphosphate reacts nonenzymatically easily with amino acids in contrast to inactive monoacetylphosphate. It does, however, not act enzymatically as acetyl donor (93). Chantrenne (34) emphasizes the need of rigorous controls for the evaluation of results obtained with the hydroxamic acid method for the determination of acyl phosphate (122).

UREA SYNTHESIS

There has been a lag period of almost ten years after Krebs' demonstration of the ornithine-citrulline-arginine cycle in tissue slice experiments but promising breaks have been obtained recently. Of particular importance appears to be the separation of the enzymatic systems involved in the ornithine-citrulline and the citrulline-

arginine phase respectively. The recent work of Borsook & Dubnoff (24), of Cohen and his collaborators (37 to 40), and of Ratner (158, 159) indicates that the ornithine-citrulline reaction is particle-bound, while the citrulline-arginine system is found in the liver extract. Both steps have now been studied separately in detail. Cohen's group has been most successful in the elucidation of citrulline synthesis. Glutamate was found to react as an initial carbon dioxide acceptor (37). In the search for a glutamic acid derivative as intermediary, carbamyl glutamic acid was tried and found to be highly active as carbamyl donor (38) to ornithine to form citrulline. The sequence thus appears to be the building up of a carbamyl group on to the amino group of glutamic acid and the transfer of this group to the amino group of ornithine. However, the transfer still requires oxygen, i.e., energy and, furthermore, the presence of free ammonia, the need of which at present is unaccounted for. The over-all reaction, ornithine to citrulline, in the homogenate depends for maximum rate on an unusually high concentration of ATP or adenylic acid, namely 2×10^{-3} molar. This is suggestive of a dependence on phosphate-bond energy. An anaerobic support of synthesis by ATP, however, could not yet be demonstrated.

The second step, the citrulline to arginine conversion, was found by Ratner to require aspartic acid as a specific amino donor (158). Acetone powder extracts were obtained from beef kidney which catalyzed the reaction, citrulline + aspartic acid + ATP → arginine + malic acid. A primary condensation product appears to be formed (159) and it is this condensation which requires phosphate-bond energy:



The condensation product subsequently is hydrolyzed to arginine and malic acid. The nature of the initial phosphorylated intermediary is undecided.

Carbon dioxide fixation in oxaloacetate.—It appears appropriate at this point to mention some recent observations on the mechanism of carbon dioxide addition to pyruvate, i.e., the Wood-Werkman reaction. Two independent pathways may exist, one coupled with hydrogenation and another one dependent on phosphorylation. Ochoa *et al.* (147) obtained a purified enzyme system from pigeon liver which catalyzes the reduction of pyruvate and

carbon dioxide to malate by reduced triphosphopyridine nucleotide (HTPN). An intermediate formation of oxaloacetate appears doubtful because a reduction of oxaloacetate by HTPN could not be obtained with the same enzyme preparation. Ochoa's reaction occurs in the absence of phosphate or ATP. The direct condensation of pyruvate and carbon dioxide to oxaloacetate was found by Utter & Wood (188) to require ATP. Only a negligible carbon dioxide fixation was observed in the absence of ATP. These results were confirmed by Vennesland *et al.* (190), who excluded the possibility that ATP may serve to form TPN (cf. 2).

CARBON TO SULFUR BOND

Evidence accumulated during the last few years suggests a relation between synthesis and splitting of the carbon-sulfur bond and phosphate bond transfer. The problem deserves attention but still needs some clarification. Some time ago Binkley (16) extending observations made in du Vigneaud's laboratory (18) studied the splitting of cystathionine in liver extract and found that in thoroughly dialyzed extracts a cleavage only occurred on addition of zinc salt and ATP. The phosphate balance seemed to indicate a quantitative relationship between cystathionine cleavage and a conversion of easily hydrolyzable into difficultly hydrolyzable phosphate; a formation of phosphohomoserine was assumed. It seems, however, noteworthy that in crude liver extracts the cystathionine splitting appears to proceed without addition of ATP (18). Extensive cleaving of cystathionine and related compounds with crude liver extract without addition of ATP was recently confirmed by Anslow & du Vigneaud (3).

Binkley reports now (17) on an ATP breakdown in liver extract prompted by methionine. Release of inorganic phosphate is only apparent, the phosphate being bound in a labile form and a calcium salt of a phosphorylated methionine appears to have been isolated. A similar compound was prepared from pyrophosphoric acid and methionine. Borsook & Dubnoff (26) confirm Binkley's thesis of a need of activation for disruption of the carbon-sulfur bond. They became aware of a difference in the mechanisms of methylation from choline and from methionine, respectively. Choline yields its methyl in homogenates in the absence of oxygen and with oxidation inhibitors present. Methionine, however, transfers methyl to guanidoacetic acid or nicotinic acid only in the

presence of oxygen, presumably because of need of activation. In a detailed study of this oxygen effect (25) on methyl transfer from methionine in liver homogenate they find that ATP, but not adenylic, will considerably enhance the methyl transfer, particularly in oxygen. Anaerobically an effect of ATP persists but is restricted to about 15 per cent of the aerobic transfer. Phosphoguanidoacetic acid and oxidation products of methionine could be excluded as intermediaries. A phosphorylated condensation product of methionine and guanidoacetic acid is suspected. Such a phosphorylated intermediary most likely would be a sulphonium derivative.

RESPIRATORY PHOSPHATE BOND GENERATION

Most of the synthetic reactions mentioned in the previous paragraphs were studied in homogenates. The progress in this field is largely due to improvement in the handling and especially in the washing of homogenates. Through the work of Cohen (40, 43), Lehninger (113), Potter (153, 153a), and, particularly, Green (70, 71, 72) and their associates, the routine procedure of preparing substrate-free, washed homogenates has been considerably perfected. Of practical and theoretical importance appears to be the observation that the respiratory and the energy-transforming functions reside almost exclusively in the mitochondria fraction (97), as isolated by the procedure of Hogeboom *et al.* (80) by a differential centrifugation in 30 per cent sucrose solution. The metabolic function of the particle residue remains still fairly well preserved with partly damaged mitochondria structure as obtained by washing with saline solution. Nevertheless, the preservation of relative structural integrity appears to be required for the functioning of the oxidation system and particularly for its coupling with phosphorylation.

The requirement of inorganic phosphate, magnesium, and adenylic acid for fatty acid oxidation through the citric acid cycle was restudied by Lehninger (113) with washed liver residue. He now reports equivalence of ATP and adenylic acid, while in his earlier experiments (111, 112) with crude homogenates ATP appeared required. With his washed liver preparations no appreciable phosphate fixation occurred even if glucose was added as a phosphate acceptor. However, using radioactive phosphate as an indicator, phosphate exchange into various ester fractions and

respiratory activity was found to be largely parallel. The ability to esterify phosphate is lost, however, on aging at 2°C. for two days without abolishing or even impairing respiratory activity.

A dissociation of respiratory hydrogen transfer and phosphorylation by the action of dinitrophenol was demonstrated by Loomis & Lipmann (126). Their experiments were done with a washed kidney residue similar to that described by Green *et al.* (71). To promote phosphate fixation yeast hexokinase, glucose, and fluoride were added in addition to the usual complements. With this system normally an average fixation of 2.3 moles of phosphorus per atom oxygen were found, without applying a correction for the phosphate leak, which bears out Ochoa's earlier results (146) of approximately three moles of phosphorus per atom oxygen with application of a leak-correction and contradicts the prediction of less than two phosphorus fixable per atom oxygen by Ogston & Smithies (148). Ten thousandth molar *m*-dinitrophenol and other nitro- or halogen-phenols in similar concentrations abolish phosphate fixation with no effect or rather stimulation of oxygen consumption. The inhibitory effect on phosphate fixation runs very strictly parallel with the inhibition of arabaria egg division by nitro- and halogen-phenols reported earlier by Clowes & Krahll (36).

Significantly, dinitrophenol almost abolishes the phosphate and adenylic acid requirement for respiratory activity (125, 126). The manner by which dinitrophenol disrupts the phosphate coupling is not yet fully understood. It does, however, not act by a phosphate dissipation as does arsenate. As mentioned, dinitrophenol does not inhibit respiration. This is, however, strictly true only with di- or tricarboxylic acid as the respiratory substrate. With fatty acid or acetate, dinitrophenol completely suppresses oxidation (100, 125). With pyruvate, an inhibition is observed which may be overcome by the addition of dicarboxylic acid (125). It is concluded that acetate and other fatty acids require phosphate bond energy for condensation with oxaloacetate as does also the carboxylation of pyruvate to oxaloacetate (cf. 188, 190).

Using ferricyanide as hydrogen acceptor in the presence of kidney homogenate, yeast hexokinase and glucose as phosphate acceptor, about one mole of phosphate was found to be fixed for every two moles of ferricyanide reduced (125). Since two moles of ferricyanide are equivalent to one atom of oxygen, the

phosphate yield per hydrogen transfer is about half that for oxygen. Dinitrophenol as well as azide disrupts the coupling with phosphorylation without affecting ferricyanide reduction. Another anaerobic phosphate bond generation is reported by Hunter in the presence of kidney or liver homogenate (85). He studied the reaction between ketoglutarate and oxaloacetate, with ketoglutarate serving as hydrogen donor and oxaloacetate acting as the hydrogen acceptor. Using yeast hexokinase plus glucose as phosphate acceptor, a yield of 1 mole of phosphate per pair of hydrogen atoms transferred was found. Since no correction was made for any phosphate leaks, the actual yield may be greater than one.

While these observations indicate a relative independence of the cytochrome system, a phosphate bond generation with reduced DPN as hydrogen donor to oxygen indicates relative substrate-independence of phosphorylation as shown by Friedkin & Lehnin-ger (64).

Spiegelman *et al.* (172) have shown that the ability of yeast cells to esterify phosphate is inhibited by sodium azide together with the inhibition of adaptation; azide could not replace phosphate in the triosephosphate dehydrogenase reaction. In fact, azide like dinitrophenol (172) had no effect on cell-free glycolysis. Loomis & Lipmann (125) find with kidney homogenate that azide may lower the phosphorus:oxygen ratio. A clear-cut separation of hydrogen transfer and phosphorylation could, however, be obtained by the use of ferricyanide as hydrogen acceptor. Hotchkiss (81, 82) has found that gramicidin acts as staphylococci in the same manner as does dinitrophenol. Gramicidin similarly prevents the phosphorylation of glucose in muscle and kidney extracts without influencing respiration (82). Atabrine, methylene blue, and other dyes have dinitrophenol-like properties in their action on homogenates (125, 126). Usnic acid, which has antibiotic action on the tuberculosis bacillus, also inhibits cell division and P^{32} uptake of sea urchin eggs but has no effect on the oxygen consumption (130).

Meta- and pyrophosphate formation.—Recently renewed interest developed for the curious polyphosphate accumulation when it was found that metaphosphate would appear in yeast after a previous phosphate-starvation period, when transferred back to phosphate medium (86, 166, 192, 193). Wiame (193) has found that meta-

phosphate stains with basic dyes, especially toluidine blue; it can be demonstrated histologically in yeast cells where it had been mistaken for nucleic acid. The compound formed in yeast was identified as hexametaphosphate (194). Schmidt and his co-workers (167) independently found that fresh yeast without pretreatment will also form metaphosphate in a medium containing ammonium or potassium salt in addition to phosphate. Potassium uptake parallels metaphosphate formation. Indications are reported for the presence of two types of metaphosphate in yeast (87, 88), one soluble and one insoluble in trichloroacetic acid. Fluoride inhibits (86, 167) and azide completely abolishes metaphosphate synthesis (194). No sure indications are reported so far that metaphosphate may serve as a source of energy-rich phosphate bonds.

Earlier Mann (128) had found in *Aspergillus niger* an abundant metabolic conversion of phosphate to meta- and pyrophosphate. Recently its hydrolysis there was studied by Ingelmann & Malmgren (199). Conversion of inorganic phosphate to pyrophosphate has been reported in tissue preparation (48, 72) and an accumulation of metaphosphate was observed in mutants of *neurospora* (84).

A promising suggestion for a mechanism of pyrophosphate formation appears from a report by Kornberg (102). He observed pyrophosphate formation by the reaction of ATP with nicotinamide ribonucleotide, yielding diphosphopyridine nucleotide (DPN) and inorganic pyrophosphate. Subsequently, a split of DPN may liberate adenylic acid and nicotinamide nucleotide. The former could then be rephosphorylated to ATP and continue to react with the latter. In this manner a catalytic conversion of inorganic to pyrophosphate may be obtained.

SYNTHESIS OF GLUCOSIDIC AND NUCLEOSIDIC LINKAGES: POLYSACCHARIDES

The branching and the priming problems are increasingly assuming a general importance for our concepts of biosynthesis. The mechanism of chain branching affords a first insight into the mechanism of a structuration, and the priming of polysaccharide synthesis, by polysaccharides of various chain length, may be considered a primitive instance of reduplication.

Branching.—In their work with tissue phosphorylases, the Coris (50) observed early that liver and heart extracts contain a

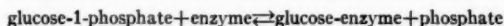
"branching factor" which complements purified muscle phosphorylase to synthesize glycogen and amylopectin. The purified muscle phosphorylase alone only manufactured amylose, a straight chain polysaccharide with an exclusive 1 to 4 linking between glucoses. The glycogen and amylopectin structures, in addition to 1-4-linked straight chains, contain branches off the main chain. At branching points a glucose-1-link forms to carbon 6 of a chain-glucose, the branch continuing then with 1 to 4 links as in the straight chain. In plant extracts, the 1-4 linking enzyme was named by Haworth *et al.* (75) the P-enzyme, while the branching factor was called the Q-enzyme. Recent observation by Peat *et al.* (150) on the transformation of amylose into amylopectin with potato extracts seemed to make it doubtful if the potato branching factor were a true phosphorylase. However, an isophosphorylase specific for 1-6 linking was isolated from potato extracts by Bernfeld *et al.* (15). The isophosphorylase in conjunction with normal potato phosphorylase converted amylose to amylopectin; phosphate was found to be essential for this reaction. In line with the above are observations by Katz *et al.* (96), who find that in purified potato extract arsenolysis of amylopectin stops at about 50 per cent degradation while amylose is completely decomposed to glucose. The term arsenolysis (54) was introduced for the splitting of polysaccharides by phosphorolytic enzymes with arsenate replacing phosphate. With arsenate, free glucose forms instead of glucose-1-compound; primarily formed arsono-1-glucose appears to decompose spontaneously. Sometimes the use of the arsenolysis is advantageous in studies on phosphorolysis because the reaction product does not pile up.

A parallel between phosphorolytic and hydrolytic enzymes has been elaborated recently (79, 179). The β -amylase is comparable to purified phosphorylase; both attack only 1-4 linkages. The α -amylase is comparable to purified phosphorylase plus branching factor; it attacks both, 1-4 and 1-6 linkages.

Swanson & Cori (178) have studied in greater detail the priming requirements of muscle and potato phosphorylase. By using the products of controlled degradation by acid and enzyme hydrolysis of glycogen, amylopectin, and Schardinger dextrans, they show that muscle and potato enzyme require different particle sizes for activation corresponding to a rather large unidentified number and to 5 to 6 glucose units respectively. Noteworthy is the activity

as primer for both muscle and potato enzyme of glucosan (178), a bacterial polysaccharide built up exclusively by a 1-6 linking of glucose units. This polysaccharide is able to prime the enzymes in spite of their inability to decompose the glucosan. The priming effect is considered due to the emerging of free glucose end groups from a larger particle, and the linking of the glucose in this particle seems to be not of decisive importance.

Synthesis of sucrose.—A new interpretation of the action of sucrose phosphorylase is suggested by recent observations by Doudoroff, Barker & Hassid (55). Using radioactive phosphate they found that glucose-1-phosphate, even in the absence of fructose as acceptor, exchanged rapidly with inorganic phosphate. This suggests that glucose-1-phosphate reacts reversibly with the enzyme, forming a glucose-enzyme compound:



With this interpretation a main valence link between glucose and enzyme is postulated which is equivalent to a glucosidic linkage. This opens some rather interesting possibilities for the explanation of enzymatic group transfer in general. A somewhat similar observation was earlier reported in experiments with acetyl-phosphate (120), which in bacterial extracts very rapidly exchanges with inorganic phosphate. A formation of an energy-rich acetyl-enzyme compound appears now as an interesting interpretation.

Further experiments indicate that sucrose (56) and glucose-1-phosphate may react with the same enzyme to form a glucose-enzyme bond. The sucrose analogue, glucosidosorbose, was obtained directly from sucrose and sorbose in the absence of phosphate. Sucrose phosphorylase may, therefore, be considered more broadly as a transglucosidase through which glucose is transferred to a suitable acceptor (74). In muscle and potato phosphorylase the mechanism of glucosidic bond transfer involved appears to be different and more complex. With these enzymes under similar conditions Cohn & Cori (44) could not detect exchange between tagged phosphate or tagged glucose and glucose-1-phosphate or glycogen.

Dextran and polysaccharide synthesis from sucrose.—Hehre (77), who discovered the enzyme in *Leuconostoc mesenteroides* which converts sucrose into dextran and free fructose, describes now a

conversion of sucrose without intermediation of phosphate into a glycogen-like polysaccharide with liberation of fructose in *Neisseria* (77, 78, 79). However, it should be recalled that for the initial energy lift from mono- to disaccharide somewhere down the line phosphate bond energy had been incorporated (57).

Nucleotide synthesis.—The work of Kalckar on the enzymatic synthesis of purine nucleosides was published extensively (90), including his elaborate methods of spectrophotometric distinction of members of the purine series. Klein's reactivation (99) of dialyzed nucleosidase with phosphate or arsenate is recalled, and the mechanism of this reactivation now explained by identifying nucleosidase as a nucleoside phosphorylase. A role of ribose-1-phosphate in nucleotide synthesis had been suggested in an earlier review of this series (116).

The phosphorolysis of inosine to ribose-1-phosphate and hypoxanthine was studied in greater detail; an analogous reaction occurs with guanine. The equilibrium is rather far on the side of nucleoside synthesis. A purified enzyme from rat liver was used. Some 20 mg. of barium ribose-1-phosphate were isolated and characterized as a nonreducing aldopentose with phosphate attached to the aldose group. Ribose-1-phosphate is considerably more acid-labile than glucose-1-phosphate. The phosphate method of Lowry & Lopez (127) must be used to avoid decomposition.

Using Kalckar's liver preparation and a preparation of calf's thymus, Manson & Lampen (129) find in the presence of arsenate a rapid formation of reducing sugar [cf. also Klein (99)] and with phosphate, a slow formation of stable organic phosphate identified as desoxyribose-5-phosphate. These results suggest a phosphorolysis of desoxyriboside to desoxyribose-1-phosphate, followed by transformation to the 5-ester. Kalckar (89) obtained recently the direct synthesis of desoxyriboside from desoxyribose-1-phosphate and hypoxanthine.

Present investigations (53, 156, 165, 191) have extended Dische's earlier findings (52) that the ribose of adenosine may be converted into triose and hexose phosphate. Apparently ribose-5-phosphate is formed through phosphorolysis and mutase systems and splits into triose phosphate and a two-carbon compound (53, 156). Racker (156) has partially purified an enzyme in bacterial extracts that converts ribose-5-phosphate to glycolaldehyde. A condensation of triose phosphate and glycolaldehyde to a pentose

phosphate was obtained with crystalline aldolase from rabbit muscle (156). The compound was isolated and appears not to be identical with ribose-5-phosphate. Schlenk & Waldvogel (165, 191) recovered on the degradation of purine nucleosides about 50 per cent of the ribose as hexose-6-phosphate. A condensation of the two-carbon compound derived from pentose to hexose is reported by Dische (52) in human erythrocytes.

KINASES

The term kinase is now generally used for a family of phosphate transferases from ATP to a variety of compounds. The kinases frequently initiate a metabolic chain and thus determine the over-all rate. Furthermore, presence or absence of a matching kinase may allow or forbid a sugar variety to enter into the general metabolic pathway. The modification of kinase activity emerges as responsible for a varied biological symptomatology, e.g., for hormonal regulation and for the adaptation to the use of particular sugars (185).

Hormonal effects on hexokinase.—The observation by the Coris and their group (49, 154) on the antagonistic effect of pituitary extract and insulin on tissue hexokinase has naturally solicited considerable interest and discussion. In their more recent extensive publication Colowick, Cori & Slein (46) report two main types of experiments: (a) using hexokinase preparations from skeletal muscle of alloxan diabetic rats, and (b) using normal brain hexokinase. In very freshly prepared muscle extracts of alloxan diabetic rats an initial inhibition was observed which was relieved by addition of insulin. Amorphous insulin (Lilly), 10 to 20 μ g, corresponding to 0.4 to 0.8 units per cc. was used. The inhibition disappeared very rapidly on storage even at 0°C. This initial inhibition was frequently exaggerated or even sometimes provoked by the addition of adrenal cortex extracts (ACE) (Upjohn). The magnitude of inhibition was of considerable variation. Three batches of alloxan diabetic rats of 7, 8 and 15 animals each showed average inhibitions of 51, 34, and 3 per cent, respectively. The inhibition is attributed to a preponderance of hypophysis factor present in the diabetic tissue. In the second series a normal, partially purified beef brain hexokinase was used as the enzyme. The antagonistic effect of anterior pituitary, generally fortified by ACE, and of insulin was studied. The pituitary extract sometimes alone and some-

times only in conjunction with the ACE effected a 20 to 75 per cent inhibition in the positive cases and was abolished by insulin (0.8 units per cc.) which by itself had no effect. Alkali-treated insulin was inactive. Altogether 23 inactive and 42 active preparations were obtained from beef and sheep pituitaries. ACE action was apparently only accessory to pituitary. The difficulties of obtaining consistent inhibition is attributed to the lability of the active principle. When active it was, however, consistently counteracted by insulin.

Confirmatory results were reported by Reid *et al.* (160), who obtained a depression of hexokinase activity by pituitary extract and a reactivation by insulin. Similar results are reported by Broh-Kahn & Mirsky (28), who, however, saw an insulin reversible inhibition also with addition of a spleen extract. An insulin reversible inhibition of hexokinase was furthermore reported by Kun (109) to occur with meningococcal antitoxin. Stadie *et al.* (173) were unable to produce an effect of insulin on hexokinase of alloxan diabetic rats but used a different strain of rats.

The glucose turnover by isolated rat diaphragm and its insulin stimulation was discovered several years ago by Gemmill & Hamman (67, 68) and is used now by Cori & Krahl (105) as an indicator for hexokinase activity in the tissue. The effect of alloxan diabetes (depressive), adrenalectomy (neutral), and hypophysectomy (stimulatory) (106) on glucose utilization of the isolated diaphragm was mapped. The lowered glucose utilization of alloxan diabetic muscle was normalized by subsequent adrenalectomy. In all these cases, also after hypophysectomy, a normal insulin effect was observed. The diabetic diaphragm was relatively little stimulated by insulin. Perlmutter & Greep (151) measured glycogen formation as well as glucose utilization in diaphragm slices. In confirmation of earlier experiments by Gemmill they find glucose disappearance generally larger than glycogen formation. Hypophysectomy caused an increase in glucose disappearance with 0.3 per cent but not with 0.1 per cent glucose in the medium. The insulin effect was unaffected by hypophysectomy. The insulin effect on rat diaphragm was also studied extensively by Riesser (162). Besides confirming and extending earlier observations, he reports that epinephrine reliably counteracts the effect of insulin on the synthesis of glycogen.

A number of publications from Young's laboratory (160, 197) indicate attempts to reconcile the difference in the stabilities of the rather sturdy diabetogenic factor and the very labile hexokinase-inhibitory activity. They obtained an insulin effect on the hexokinase from animals injected with diabetogenic pituitary extract, which, however, showed no *in vitro* activity on hexokinase. The possibility of an *in vivo* reactivation was proposed and, in partial confirmation, preincubation of isolated rat diaphragm with diabetogenic factor is reported (149) to cause an inhibition of the insulin effect on glucose utilization.

Cori (49) has expressed the expectation that insulin may have other points of action besides on the hexokinase system, and presented unexplained effects of insulin on liver respiration. Bloch *et al.* find (21) a stimulatory effect of insulin on pyruvate metabolism. These findings coupled with the earlier results of Krebs & Eggleston (107), Stare & Baumann (175), and Rice & Evans (161) suggest a role of insulin in the aerobic phase of metabolism.

The stimulatory effect of insulin (Lilly) on glycogenolysis in rabbit liver slices (177) apparently is due to a contaminant since it is not abolished by alkali or cysteine treatment. The phosphorylase stimulating factor is related to the hyperglycemia principle of the alpha cells of the pancreas (76).

Chiu & Needham (35) report that adrenal cortical hormone inhibits glycogenolysis in liver slices and increases synthesis of glycogen from lactate, pyruvate, or glucose. It is proposed that the hormone acts on the oxidative generation of ATP. Desoxycorticosterone also increases glycogen formation from pyruvate and lactate.

Separation of gluco- and fructokinase.—In contrast to yeast (110) there appear to exist in animal tissue two different enzymes for the phosphorylation of glucose and of fructose. A specific fructokinase was isolated from rabbit muscle by Cori & Slein (51). Meyerhof & Geliazkova (134) differentiated the fructo- and gluco-kinase by an indirect analysis in brain homogenates. Amidone (73) and adrenochrome (137) inhibit brain glycolysis, apparently by their action on the hexokinase and fructokinase systems.

Colowick (45) reports that there is present in crude muscle extract an unidentified heat stable, dialyzable organic substance which stabilizes glucokinase but which apparently is not an es-

sential component of the hexokinase system. The stabilizing effect had previously been mistaken for an activation by various substances such as guanine and reduced DPN.

Phosphofructokinase.—Taylor (182) has purified muscle phosphofructokinase, the enzyme which catalyses the reaction $\text{ATP} + \text{fructose-6-phosphate} = \text{ADP} + \text{fructose-1,6-diphosphate}$. The isolated enzyme requires magnesium for activity. Utter (186) has reported that inactivation of glycolysis in brain homogenates may be attributed to an inactivation of phosphofructokinase, which is very sensitive to a pH of 6.4 or lower. The system can be reactivated by addition of boiled liver homogenate. Colowick (45) has also indicated the instability of phosphofructokinase.

A spectrophotometric method for the determination of phosphofructokinase activity has been developed by Bücher (30) and by Racker (155), making use, respectively, of reduction or oxidation of DPN by way of a chain of enzyme reactions. Bücher (30), apparently by mistake, designates reversibility to phosphorylation of hexosediphosphate by ATP, which is certainly an irreversible reaction.

GALACTOKINASE AND GALACTOSE ADAPTATION

Trucco *et al.* (185) have made notable contributions towards the understanding of yeast adaptation to galactose. From galactose-adapted brewers' yeast a galactokinase has been isolated which specifically transfers phosphate to galactose; pure yeast hexokinase had been known not to phosphorylate galactose (110). The product of galactose phosphorylation was identified as galactose-1-phosphate. Such had been earlier found by Kosterlitz (103) to accumulate in rabbit liver on feeding of galactose. He showed also that this ester was fermented by galactose-adapted yeast whereas galactose-6-phosphate was not attacked. Kosterlitz already suggested a galactokinase in liver forming galactose-1-phosphate. Bacilia (8) has now confirmed this suggestion.

The direct phosphorylation of an aldose in the 1-position is very remarkable and so far a unique phenomenon. Some preliminary work indicates a direct enzymatic transformation of galactose-1-phosphate into glucose-6-phosphate (185). This transformation is carried out by preparations of *Saccharomyces fragilis*, an organism which has no galactokinase capacity (31). However, the same *S. fragilis* (163) and other strains of yeast, as well as some

lactobacilli (168), are known to ferment lactose although unable to utilize galactose. This behavior suggests that a phosphorolysis of lactose to galactose-1-phosphate and glucose takes place in these lactose-utilizing strains.

Phosphate transfer by phosphatases.—One of the most interesting observations in this field is the transphosphorylation from aryl phosphate to various alcohols with citrus fruit phosphatase which was discovered by Axelrod (6). Using nitrophenylphosphate (1 per cent) as donor and methanol (22 per cent) as acceptor, pure barium monomethylphosphate was isolated. The transfer reaction is followed by comparing aryl and phosphate liberation, the aryl liberation becoming relatively increased with increasing addition of acceptor alcohol. After a certain time a partition equilibrium appears to be established. Transfer was found with several but not all fruit phosphatases and with urine phosphatase. No reaction could be observed with alkaline kidney phosphatase. Adenylic acid as a participant in such a transfer was excluded. It was furthermore observed (7) that on transfer the phosphate radical does not mix with inorganic phosphate using radioactive phosphate as indicator. Appleyard (4) made confirmatory observations with prostate phosphatase.

Phosphate transfer on the energy rich level.—The enzyme which catalyzes the reversible transfer of acyl phosphate from phosphoglyceryl phosphate to ADP was crystallized by Bücher from Lebedev juice (30). Magnesium is required for the activity of the enzyme. The equilibrium is much in favor of the formation of ATP (about 90 per cent).

Kubowitz & Ott (108) have crystallized the enzyme which transfers the enol-phosphate of phosphopyruvate to ADP. Magnesium ion is apparently required for this system. An indirect spectrophotometric procedure was developed for the determination of the enzyme. Meyerhof & Wilson (139) report that phlorhizin inhibits the activity of the phosphopyruvate transferase. As yet, there is no good data on the equilibrium of this transfer reaction.

ADP-phosphomutase (myokinase).—Colowick & Kalckar (47) isolated a muscle enzyme which catalyzes the dismutation of 2 ADP to ATP and adenylic acid, and thus converts the energy-rich phosphate of ADP into terminal phosphate of ATP. This enzyme myokinase is heat stable and no analogous enzyme was ob-

served in other tissues. Recently Kotelmikova (104) isolated an enzyme from liver which catalyzes the transfer of phosphate from ADP. The liver enzyme is much less stable to acid and boiling than is the muscle enzyme. A similar transfer system has also been found in *E. coli* extracts (95). It is of interest to mention that in crude acetone powder extracts of pigeon liver (95), ADP serves as a better source of energy rich phosphate than ATP, apparently because it generates continuously ATP, being itself resistant to adenosinetriphosphatase. Kotelmikova (104) recommends the name ADP-phosphomutase be used rather than myokinase.

GLYCOLYSIS

The rate of ATP turnover as a regulator for the rate of metabolism, particularly fermentation, has attracted considerable interest lately. Engelhardt & Seitz (61) confirmed Meyerhof's findings (133) that the ATP-drain by potato apyrase (enzyme splitting both easily hydrolysable P of ATP) increases fermentation of hexosediphosphate by yeast maceration juice. With sufficient apyrase the yeast juice ferments glucose at the same rate as live yeast, and under these conditions the fermentation obeys the Gay Lussac equation, and not the Harden-Young equation for cell-free extracts. Excessive amounts of apyrase, however, stop fermentation because no ATP is left for the phosphorylation of glucose.

Meyerhof & Wilson (138, 139) find, due to the activity of adenosinetriphosphatases, a higher turnover of hexosediphosphate in brain homogenates than in brain extracts. Earlier work (65) which indicated poor activity of hexosediphosphate in brain extracts was misleading and led to the wrong conclusion that it was not on the main path of glycolysis. A study of hexose monophosphate fermentation gave similar results (61). The regulation of the concentration of ATP appears to be an important consideration in the maintenance of metabolism in the intact cell.

Novikoff, Potter & LePage (145) have shown that five- to eight-day chick embryo homogenates glycolyze hexose phosphates and glucose. These results seem to rule out previous concepts concerning nonphosphorylating glycolysis in embryonic tissue.

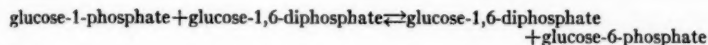
ENZYMES OF THE GLYCOLYTIC SYSTEM

Phosphoglucomutase (glucose-6-phosphate \rightleftharpoons glucose-1-phosphate).

—Najjar (144) has crystallized the enzyme from muscle. The

crystalline enzyme is active only in the presence of magnesium ion and cysteine. Fluoride appears to form a magnesium-fluoride-organic phosphate complex which competes with magnesium for the enzyme. As in the case of enolase, inorganic phosphate increases the inhibition of fluoride. Schlamowitz & Greenberg (164), through the use of radioactive glucose have ruled out an enzyme phosphate intermediate in the mechanism of phosphoglucomutase action; Meyerhof *et al.* (136) have earlier eliminated the possibility of inorganic phosphate being involved.

Caputto *et al.* (31) have found a heat stable cofactor for phosphoglucomutase in preparations obtained from two different strains of yeast. This factor is also required for the conversion of galactose-1-phosphate into a reducing ester in *S. fragilis*. Partial purification and the synthesis of active although impure material yielded presumptive evidence for glucose-1,6-diphosphate. The coenzyme is assumed to function as intermediary in the following manner:

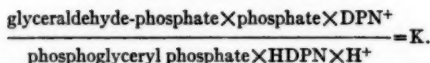


In this way a reaction thought to be an intramolecular phosphate transfer appears now as an intermolecular transfer reaction. These observations by Leloir *et al.* (114) suggest a rather remarkable and unexpected mechanism.

Aldolase (*phosphoglyceraldehyde + phosphodihydroxyacetone* \rightleftharpoons *fructosediphosphate*).—This enzyme which constitutes about 10 per cent of the total soluble protein of muscle has been isolated from rabbit skeletal muscle (183). The enzyme appears to contain an excess of basic over acidic groups and tends to bind anions such as phosphate (189).

Glyceraldehyde phosphate dehydrogenase.—Taylor *et al.* (184) have found now that crystalline glyceraldehyde phosphate dehydrogenase contains a definite amount of bound DPN which is not removed by prolonged dialysis. Only by treatment with intestinal phosphatase and norite could cozymase be fully removed. Significantly the DPN-free protein does not crystallize but crystallization is induced by readdition of DPN.

Meyerhof & Oesper (135) have found the oxidation reaction of fermentation to represent a true thermodynamic equilibrium with respect to all reactants involved:



Evidence is presented that an unstable phosphate addition product of glyceraldehyde-phosphate forms only in the presence of the oxidizing enzyme and DPN. A rather specific binding of phosphate by the crystalline enzyme has been observed by Velick (189). The enzyme exhibits the properties of a basic protein. An inhibition of brain glycolysis by ferrous sulfate has been found by Racker & Krimsky (157) and is assumed to be localized in the phosphoglyceraldehyde oxidizing system.

ADENOSINETRIPHOSPHATASE AND MYOSIN

An adenosinetriphosphatase is generally understood to be an enzyme which hydrolytically decomposes ATP. Through such hydrolysis the energy of the phosphate bond is obviously dissipated. It appears, however, pertinent that an ATP-dependent synthesis may easily be confused with ATP hydrolysis. We find for example that in pigeon liver extract acetate induces a split of ATP but only to give rise to acetoacetate (169) and probably other condensation products. It may well happen that what is in fact an energy transfer may easily appear as mere ATP hydrolysis as long as the pathway of the dependent synthetic reaction is not understood. It is our impression that the ATP split with myosin will eventually be found to be an energy transfer reaction. The finding by Bailey & Perry (9, 10) that the intactness of the same grouping, namely sulfhydryl groupings, is essential for the occurrence of a linking from myosin to actin as well as for the "adenosinetriphosphatase" activity of myosin may be a step forward in the indicated direction. A further relevant observation is reported by Szörényi & Chepinoga (181), who found that phosphate split off from ATP by myosin adenosinetriphosphatase appears partially to be bound to myosin. Inorganic phosphate when added to myosin or actomyosin is freely diffusible whereas part of the ATP-split phosphate is not; about 10 to 15 mg. of phosphate were bound per gm. myosin. If the molecular weight of myosin is assumed to be 100,000, this would be equivalent to about 50 moles of phosphorus per mole of myosin.

The question of whether the adenosinetriphosphatase activity

is inherent in the myosin molecule or absorbed on the muscle protein has been further explored. Polis & Meyerhof (152) report enrichment of myosin adenosinetriphosphatase activity by precipitation with a basic lanthanum salt followed by elution with a potassium chloride solution containing cyanide and ATP. Gilmour (69) has found that adenosinetriphosphatase activity can be separated from insect myosin by washing and repeated precipitation.

Mommaerts & Seraidarian (141) have concluded that myosin adenosinetriphosphatase is responsible for only a small part of inorganic phosphate liberated in contracting muscle. This may be related to the discovery by Kielley & Meyerhof (98) of a magnesium activated adenosinetriphosphatase which can be extracted with dilute alkaline salt solution from muscle. The enzyme is inhibited by calcium whereas the myosin adenosinetriphosphatase is stimulated by calcium and inhibited by magnesium (27, 140, 141). It is present in muscle in approximately the same amounts as the myosin adenosinetriphosphatase and splits only one phosphate group of ATP (98). The reader is referred to Szent-Györgyi's stimulating book (180) for details on the actomyosin reaction.

ENERGY SOURCE FOR BIOLUMINESCENCE

ATP has been found by McElroy (131) to promote luminescence in the firefly (*photinus pyralis*) extracts. The amount of light emitted in fresh extracts of the firefly is dependent on the concentration of ATP, ATP-splitting activity and luminescence being closely parallel. A divalent ion, such as magnesium, manganese, or cobalt, seems to be necessary for the ATP stimulated light reaction. Oxygen is also required.

Luciferin preparations from the crustacean cypridemia or the firefly contain labile phosphate groups which were split during the light emitting reaction (131, 132). The enzyme luciferase, which attacks the luciferin substrate, apparently is responsible for the liberation of phosphate. It is possible that the labile phosphate in the luciferin is the immediate source of energy for the light reaction and that ATP acts by regenerating the labile phosphate of the luciferin. The interesting results of McElroy and his co-workers promise to give insight into another important function of phosphate bond energy.

PHOTOSYNTHESIS AND PHOSPHATE TURNOVER BY THE
USE OF RADIOACTIVE PHOSPHATE

Gest & Kamen (66) and Aronoff & Calvin (5) studied the effect of light on phosphate turnover. Gest & Kamen, using living algae and *Rhodospirillum rubrum*, made a careful study of phosphate requirement and turnover in these organisms. Using the trichloroacetic acid insoluble phosphate fraction as the most reliable indicator, they find an almost tenfold increase of phosphate uptake and, likewise, increased phosphate turnover in the light. The data are considered suggestive but inconclusive with regard to phosphate participation in the process of photosynthesis. Aronoff & Calvin's results are also undecisive. However, Benson & Calvin (14) report to have found with C^{14} as indicator phosphoglyceraldehyde and phosphoglyceric acid among the primary products of photosynthesis [Brown *et al.* (29)].

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CARBOHYDRATE METABOLISM¹

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The present review deals chiefly with carbohydrate metabolism in higher animals. Certain areas have been treated in some detail, and as a consequence it has been necessary to do little more than mention many papers, and to omit completely many others.

The increased interest in the study of effect of hormones and inhibitors on the glycolytic system, using *in vitro* methods is noteworthy. Great impetus was given to these studies by the observation that extracts from the pituitary gland and adrenal gland influence the hexokinase reaction and that this effect is counteracted by insulin. Furthermore, with improved methods of isolating crystalline enzymes it has become possible to study more accurately the enzyme systems. By addition of purified enzymes under controlled conditions to a tissue preparation a selected enzyme may be established as the "pace maker" and the activity of the "pace maker" enzyme may then be determined. Enzymes are becoming stock reagents in biochemical laboratories. In addition, the spectrophotometric method of enzyme assay as introduced by Warburg and collaborators has provided an exceedingly useful tool for rapid study of reactions. In fact, the Beckman spectrophotometer is replacing the respirometer as the favorite instrument for enzyme investigation.

The problem of enzyme assay in crude materials is difficult, however, and attention must be given to all factors which control the reaction rate if erroneous results are to be avoided. Although the time for the application of enzyme chemistry to the field of clinical medicine is at hand, undue optimism is not warranted because there is still much to be learned before the step from *in vitro* systems with purified enzymes to complex systems with tissue extracts, and to the intact animal, can be made with assurance.

ANAEROBIC GLYCOLYSIS

The reactions of anaerobic glycolysis are probably the best understood of any series of interlocked biochemical reactions. As a

¹ This review covers the period from October, 1947 to November, 1948.

consequence many studies have been undertaken of the effect of disease, hormones, and inhibitors on this series of reactions. However, the difficulty of such study is well illustrated by the investigations of Racker & Krimsky (1, 2). These workers have been attempting to determine whether or not infection by neurotropic viruses inhibits glycolysis of brain. They have obtained effects with partially purified viruses and have traced the effect to the iron content. With coenzyme-fortified homogenates of normal mouse brain, it has been found that glycolysis of glucose is inhibited after preliminary incubation with ferrous ion, but glycolysis of hexosediphosphate (HDP) is not inhibited. It thus appeared that the inhibition was at the phosphorylation of the glucose to HDP. Racker & Krimsky (2) have now presented evidence that this is not the direct site of inhibition. The inhibition is suggested to be at the glyceraldehyde phosphate dehydrogenase. Addition of crystalline phosphoglyceraldehyde dehydrogenase to iron-inhibited brain restored the activity on glucose. Furthermore, when adenosinetriphosphate was generated by addition of Lohmann's enzyme and creatine phosphate, activity was likewise restored. The explanation on the basis of inhibition of glyceraldehyde phosphate dehydrogenase is complicated by the fact that the dehydrogenation, as such, is part of both the HDP and glucose systems so that inhibition of HDP as well as glucose might be expected by ferrous ion. Although the restoring effect of addition of glyceraldehyde phosphate dehydrogenase appears to locate the site of inhibition, a direct measurement of the triosephosphate dehydrogenation reaction in iron-inhibited brain would seem desirable as further evidence. Racker & Krimsky have presented a helpful discussion of the problem of enzyme assay in their paper (2).

The comparison of inhibitors on glucose and hexosediphosphate glycolysis has frequently been used as an indication of the activity of an inhibitor on hexokinase. As noted above, the fact that glucose fermentation is inhibited and fructose-6-phosphate and HDP are not, is by no means conclusive evidence that hexokinase is the direct site of the inhibition. Any factor which alters the concentration of ATP will influence the hexokinase reaction; thus glucose utilization may be retarded by an overactivity of adenosinetriphosphatase or by a decrease in the efficiency of coupled phosphorylation. On the basis of rates of glucose and HDP glycolysis Greig (3) has indicated that hexo-

kinase is inhibited by amidone and Meyerhof & Wilson (4) that capryl alcohol and phenylurethane inhibit hexokinase and phosphohexokinase. However for definite establishment of this focus of inhibition, it appears that more extensive studies are needed. The evidence that adrenochrome [Meyerhof & Randall (5)] inhibits hexokinase and phosphohexokinase is more conclusive since a direct measurement was made of the glucose utilized and an excess of ATP was demonstrated at the end of the experiment.

As noted by Broh-Kahn & Mirsky (6) the determination of hexokinase activity by measurement of changes in seven-minute phosphate is frequently unreliable. A determination of the glucose utilized is a more direct method than either the respirometer or phosphate determination methods.

Meyerhof & Wilson (4) have observed that glucose turns over faster in rat brain slices and homogenates than does fructose. They have now been able to duplicate this observation with extracts from brain through use of a low concentration of ATP. A possible explanation is that there are two hexokinases, fructohexokinase and glucohexokinase and that these two enzymes may differ in their affinities for ATP.

The mechanism of the conversion of glucose-1-phosphate to glucose-6-phosphate has been studied by Schlamowitz & Greenberg (7) using C^{14} -glucose and a phosphoglucomutase which was free from phosphorylase and hexose isomerase. Meyerhof *et al.* (8) had previously shown with P^{32} that there is no exchange of esterified phosphate with inorganic phosphate during the shift of organic phosphate from the 1 position to the 6 position of glucose. Schlamowitz & Greenberg have now shown that there is likewise no exchange of unesterified C^{14} -glucose with esterified glucose during this conversion. The phosphate transfer is therefore believed to be of an intramolecular nature and it is proposed that a diester monophosphate may be formed between the 1 and 6 positions of the glucose which is then split at the 1 position to give glucose-6-phosphate.

The findings of Leloir *et al.* (9) are interesting in relation to this suggestion since they propose glucose-1,6-diphosphate as an intermediate. In studying a coenzyme of yeast phosphoglucomutase (10) they found the coenzyme to concentrate along with HDP but the coenzyme was not HDP since after treatment with alkali the Seliwanoff reaction for fructose became negative yet the

coenzyme activity remained. The coenzyme could be synthesized in poor yield by reaction of 1,6-dibromotriacetylglucose and silver phosphate and also enzymatically with glucose-1-phosphate and ATP using an enzyme from yeast. Glucose, fructose, glucose-6-phosphate, fructose-6-phosphate and fructose-1-phosphate could not be substituted for the glucose-1-phosphate in this synthesis.

It is rather surprising to find that muscle and potato phosphorylase which catalyze reactions very similar to sucrose phosphorylase apparently utilize mechanisms that are different. Whereas sucrose phosphorylase catalyzes (11) a rapid exchange of P^{32} between inorganic phosphate and glucose-1-phosphate in the absence of fructose, it has been found by Cohn & Cori (12) that muscle and potato phosphorylase do not catalyze such an exchange of phosphate if glycogen or starch is omitted from the system. Apparently glucose-1-phosphate must combine with the polysaccharide acceptor before its phosphate becomes labilized, whereas with sucrose phosphorylase the linkage of glucose-1-phosphate with the enzyme or coenzyme causes the glucose phosphate bond to be broken even in the absence of fructose. The glucose as attached to the sucrose phosphorylase enzyme is believed to be "active" or energy rich (11) and may then react with a number of sugars to form a variety of disaccharides (13). Muscle phosphorylase on the other hand even in the complete system of glucose-1-phosphate and glycogen does not introduce unesterified C^{14} -glucose into either the phosphate ester or the synthesized glycogen (12). Furthermore in the complete system there is no exchange of phosphate with adenylic acid.

Muntz (14) has studied the effect of potassium and ammonium ions on the fermentation of glucose by yeast maceration juice and has concluded that either of these two ions is necessary for the conversion of hexosemonophosphate to hexosediphosphate. When a mixture of glucose and hexosediphosphate was fermented in the absence of potassium ion and ammonium ion the HDP was fermented to alcohol and carbon dioxide but the glucose was not. That part of the glucose which acted as a phosphate acceptor was converted only as far as the monophosphate esters. On the other hand, when potassium ion or ammonium ion was added, fermentation of glucose to carbon dioxide and alcohol occurred and that part of the glucose which was esterified by acting as a phosphate acceptor was converted not only to monophosphate esters

but in substantial amount to HDP. Fermentation and formation of HDP from glucose could in this case proceed because there was no longer a block at the monophosphate esters. This striking effect of potassium ion and ammonium ion should be studied further with purified enzymes so as to establish with certainty the site and mode of action of these cations.

Broh-Kahn *et al.* (15) have studied the kinetics of the conversion of glucose-1-phosphate and glucose-6-phosphate to glucose using liver extracts. They have concluded that the liver phosphatase acts largely on glucose-6-phosphate. This conclusion is in contrast to that of Ostern *et al.* (16) who proposed that glucose-1-phosphate was the immediate source of glucose in the liver. The combined study of the effect of inhibitors and of the kinetics as presented by Broh-Kahn *et al.* appears to support their conclusion.

Spiegelman, Kamen & Sussman (17) have continued their studies on the action of azide on the metabolism of yeast cells and have suggested that azide replaces the acyl phosphate of diphosphoglycerate. The azide thus prevents the transfer of energy rich phosphate to ATP and in this way inhibits cellular synthesis, a well-known property of azide. The fact that azide retards uptake of P^{32} as inorganic phosphate and that in the conventional schemes of anaerobic glycolysis of glucose the only uptake of inorganic phosphate is at the triosephosphate dehydrogenation, indicated to Spiegelman *et al.* that triosephosphate dehydrogenase was the point of action of azide. However, a careful study with crystalline triosephosphate dehydrogenase revealed no effect of azide on the rate of dehydrogenation nor was there evidence of replacement of the required phosphate by azide. Therefore by elimination the diphosphoglycerate-phosphopherase reaction was settled on as the likely point of action. However, no direct determinations were made of the effect of azide on the diphosphoglycerate-phosphopherase reaction. It is disturbing that azide was found to have no effect in preventing phosphate esterification in cell-free extracts at concentrations capable of complete inhibition of phosphorylation by intact cells. The significance of the *in vitro* measurements with azide on triosephosphate dehydrogenase is therefore uncertain.

That azide may not act solely by preventing formation of energy rich phosphate is shown by Reiner (18) who compared the effect of arsenate and azide on inhibition of adaptive enzyme

formation by yeast cells. The mechanism of action of arsenate is believed to be replacement of phosphate and thus prevention of formation of energy rich phosphate. If this were the source of the inhibition it would be expected that ATP would counteract the inhibition of arsenate. This was found to be the case. However, ATP did not remove the inhibition with azide so that the point of action of azide appears to be other than, or in addition to, its inhibition of formation of energy rich phosphate.

Dinitrophenol like azide completely blocks synthetic reactions. Loomis & Lipmann (19) have found that dinitrophenol inhibited uptake of inorganic phosphate. Of even greater interest is the observation that dinitrophenol stimulated oxygen uptake in the absence of added inorganic phosphate and had no effect on oxygen uptake if phosphate was present. A washed kidney homogenate was used aerobically with glutamate and fructose as substrates. Apparently the dinitrophenol replaced the requirement for phosphate and in the presence of phosphate interfered with the efficiency of transphosphorylation. This action is similar to that observed with arsenate but may not involve the same mechanism, that is, actual replacement in chemical linkages of the phosphate by dinitrophenol. Dinitrophenol may increase the availability of the limited amount of phosphate in the washed homogenate, for example, by decreasing the efficiency of formation of phosphate esters and thus not depleting the supply of inorganic phosphate.

Further studies on dinitrophenol and azide have been made by Spiegelman (20), Reiner & Spiegelman (21), and Peiss & Field (22), but space does not permit a discussion of them.

The problem of whether or not the early chick embryo utilizes a nonphosphorylating scheme of glycolysis has been reinvestigated by Novikoff *et al.* (23). Extensive chemical analysis of the chick embryo has been made for intermediary members of the carbohydrate mechanism. Glycogen, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexosediphosphate (HDP), triosephosphate, phosphoglyceric acid (PGA), phosphopyruvate, lactate, adenosinetriphosphate (ATP), adenosinediphosphate, (ADP), adenosinemonophosphate (AMP), phosphocreatine, pentose phosphate, and diphosphopyridine nucleotide (DPN) were found present in amounts compatible with the conventional glycolysis scheme. Homogenates of five to eight day embryos glycolyzed HDP, fructose-6-phosphate, glucose-6-phosphate, and glucose.

Whereas these results do not disprove the occurrence of a non-phosphorylating scheme, they show that the requirements of the generally accepted scheme are met.

There have been a number of investigations of glycolysis of a more general nature which will be mentioned but which cannot be discussed in detail. Reiner (24) has investigated the effect of age on carbohydrate metabolism of tissue homogenates of rats. With animals over two years of age, the oxygen uptake of brain fell off rapidly, whereas anaerobic glycolysis had its maximum at two to four months and then fell off slowly. There were no significant changes with age found with the liver homogenates.

A correlation of the *in vitro* glycolytic rate of heart muscle strips with the resistance of the animal to anoxia has been made by Wu *et al.* (25). In species in which the heart shows the greatest resistance to anoxia the glycolytic activity was the greatest; eel > toad > turtle > rat. Presumably glycolysis is the chief source of energy in anoxia.

The metabolism of spermatozoa and of the seminal vesicles has been studied by Mann & Lutwak-Mann (26). Fructose is produced by the seminal vesicle and is the main source of energy to the spermatozoa. Glucose can be utilized by the spermatozoa, but is absent from semen. McIlwain *et al.* (27) have found that glutamine stimulates the glycolysis of suspensions of β -hemolytic streptococci. Related compounds were not effective and the action is specific. γ -Glutamyl hydrazine inhibited the process. Soumalainen & Toivonen (28) have presented evidence that fructofuranose is the form of fructose which is fermented by living yeast. Snell *et al.* (29) have found that *Lactobacillus bulgaricus* utilizes lactose more rapidly than monosaccharides and the other disaccharides tested were not utilized. A number of synthetic β -D-galactopyranosides permitted growth including, β -n-butylgalactoside, β -phenylgalactoside, and β -methylgalactoside. The problem of direct utilization of disaccharides is considered. Rogosa (30) has studied the same problem with yeast.

The metabolism of lung from mice has been measured by using intact lobes, Phosgene had no effect on glycolysis. [Boyland & McDonald (31)]. Inhibition of hexosediphosphatase by sulfhydryl reagents and by ascorbic acid has been investigated by Walsh & Walsh (32).

The following glycolytic enzymes have been obtained in the

crystalline state or in a highly purified form. (These investigations will not be considered in detail since there are individual chapters devoted to enzymes and their properties.) Bailey & Webb (33) have crystallized yeast hexokinase and have investigated its reaction with β,β' -dichlorodiethyl sulphide using radioactive sulfur.

The crystallization of phosphoglucomutase (glucose-1-phosphate \rightleftharpoons glucose-6-phosphate) from muscle has been accomplished by Najjar (34). Cysteine and magnesium ions are required for maximum activity. It was found that inhibition by fluoride involved a complex of $[Mg][F]^2$ [organic phosphate] which presumably competes with magnesium ion for the enzyme. Crystalline aldolase (HDP \rightleftharpoons glyceraldehyde phosphate + dihydroxyacetone phosphate) has been obtained by Taylor, Green & Cori (35) from rabbit muscle. The aldolase protein could be crystallized as needles, hexagonal plates, or hexagonal bipyramids, depending upon the conditions of crystallization. The activity of each of the three forms was the same and did not change upon their interconversion. Apparently the needles correspond to the crystalline aldolase as obtained by Warburg & Christian (36) from rat muscle and are similar to the hexagonal bipyramids of Baranowski's myogen A. Stumpf (37) has prepared a purified aldolase from peas and has studied its properties.

A complete report on the crystallization and properties of glyceraldehyde phosphate dehydrogenase (glyceraldehyde phosphate \rightleftharpoons phosphoglyceric acid) from rabbit muscle is given by Cori, Slein & Cori (38). The crystalline glyceraldehyde phosphate dehydrogenase contained firmly bound DPN. The DPN could be removed by treatment with intestinal phosphatase or norit and the protein still retained its dehydrogenase activity. However crystallization of this DPN-free protein was not successful. It was necessary to add DPN and then the crystalline form was again obtained but as the DPN-protein complex [Taylor *et al.* (39)]. The amino acid composition of aldolase and glyceraldehyde phosphate dehydrogenase has been determined by Velick & Ronzoni (40).

TRICARBOXYLIC ACID CYCLE AND OXIDATION

The tricarboxylic acid cycle continues to receive the attention of numerous investigators. Little progress has been made, however, toward an understanding of the intimate details of the mechanism

of the oxidation; for example, phosphate intermediates have not been identified and the initial condensation reaction remains as obscure as ever. The principal limitation to progress appears to be the lack of a soluble enzyme system for study. Study of some of the reactions has been possible only with washed particulate portions of homogenized tissue [Hunter & Leloir (41), Lehninger (42) and Green *et al.* (43)]. Localization of the activity in certain types of particles is indicated by Kennedy & Lehninger (44) and Schneider (45), who state that oxidations of the tricarboxylic acid cycle occur with mitochondria which were obtained by homogenization of liver in sucrose and by differential centrifugation.

It is significant that Lehninger & Kennedy (46) [cf. also Potter & Klug (47)] have observed on addition of neutral salts to inactive washed cell particles that an activation of the oxidation of fatty acids occur. The salt produced a flocculation which is suggested to have produced the activation. This is reminiscent of the results of Utter *et al.* (48) who found that addition of sodium chloride to lysed preparations of *Micrococcus lysodeikticus* greatly increased respiration. They indicated that the aggregating action of the sodium chloride might be essential in orienting previously dispersed enzymes. This raises the attractive possibility that inactive soluble enzymes may be reactivated by converting them to insoluble aggregations of enzymes.

Potter *et al.* (49) find that homogenization in potassium chloride solution gives a better oxidative system than does homogenization in water. They conclude that potassium chloride treatment yields a preparation in which there is a balance between the phosphorylation and dephosphorylation reactions and that dephosphorylation is increased by homogenization in water. The system studied was the oxidation of oxaloacetate with whole homogenates from rat kidney, heart, liver, skeletal muscle, and brain. The problem of oxidative phosphorylation was investigated by measurement of phosphocreatine formation.

Green *et al.* (43) have named their cell particle preparation the cyclophorase system and have defined it as "a complex of enzymes which catalyzes the complete oxidation of pyruvic acid by way of the Krebs citric acid cycle." It should be noted that the preparation does not represent a discrete system which has been isolated, separated, or characterized, since the tissue particles will catalyze many reactions besides those of the tricarboxylic acid cycle. The

introduction of a new term of this sort which is used in a broader sense to designate the oxidations of the tricarboxylic acid cycle seems more likely to add confusion than clarity to a literature already overburdened with cryptic terminology. With their preparation, Green *et al.* (43) have studied quantitatively most of the steps of the tricarboxylic acid cycle and have presented very convincing evidence for the occurrence of a large number of the steps. As in all previous work there is little information on the intimate details of the reactions. This careful re-examination of the evidence for the cycle is a valuable contribution and provides additional information necessary for controlled studies of the tricarboxylic acid cycle and biological oxidation.

Three other papers have appeared from Green's group [Grafflin & Green (50), Knox *et al.* (51), Atchley (52)] in which the washed tissue preparations have been used to study the oxidation of fatty acids. These papers fall more properly under Fat Metabolism.

Weinhouse & Millington (53) have studied the oxidation of isotope-labeled acetic acid, $\text{CH}_3\text{C}^{13}\text{OOH}$, with yeast and have isolated citric acid from the reaction mixture. The products of the yeast were largely carbon dioxide but some C^{13} was incorporated in lipids and approximately 20 per cent of the acetate utilized was recovered as citrate. On the basis of 100 per cent C^{13} in the acetate carboxyl there was found 80 per cent C^{13} in the primary carboxyls of the citrate, 57 in the tertiary carboxyl, and the remaining carbons contained no C^{13} . The C^{13} of the respiratory carbon dioxide was 57 per cent. Weinhouse & Millington have set up theoretical calculations based on different mechanisms of breakdown of an intermediary tricarboxylic acid and have found a remarkable agreement between the observed C^{13} values and the calculated values on the assumption that an unsymmetrical acid rather than citrate is on the direct path of oxidation. The results indicate strongly that the tricarboxylic acid cycle occurs in yeast and that citrate is on a side reaction.² It is noteworthy that there was no evidence obtained that the yeast fixed carbon dioxide. The possibility of an independent mechanism of succinate formation is considered. In fact, synthesis of a C_4 -dicarboxylic acid is a neces-

² The recent proposal by Ogston [OGSTON, A. G., *Nature*, **162**, 963 (1948)] that the isotope data may be explained on the basis of a three point combination of citrate and the enzyme surface is very interesting and makes it necessary to reconsider the role of citrate in the cycle.

sity unless there is an endogenous source of C₄-acids, if citrate is formed via the tricarboxylic acid cycle.

Lynen (54) has reached a similar conclusion with yeast using 97.1 per cent deuterium-labeled acetate, and normal fumarate. In the presence of malonate, on the basis of 100 per cent deuterium in the acetate, the isolated succinate was found to contain 33.6 per cent deuterium whereas if citrate was an intermediate the deuterium would have been expected to be 25 per cent as a maximum [cf. Wood (55, p. 213) for a fuller discussion]. The theoretical value, if aconitate is the intermediate, is 50 per cent deuterium. The fact that only 33.6 per cent was observed is explained on the basis that part of the nonisotopic fumarate was oxidized to C₂-compounds and in turn entered into the condensation reaction, thus giving rise to "light" succinate. The quantitative relationship of the fumarate consumed and succinate formed are in agreement with this supposition.

Space does not permit an adequate consideration of other papers dealing with the tricarboxylic acid cycle and oxidation. Floyd *et al.* (56) have investigated the anaerobic conversion of C¹³-acetoacetic acid to citric acid by rat kidney, muscle, and brain, and find that all three tissues bring about this conversion. Krebs & Eggleston (57) with nonisotopic methods present evidence that citrate is not an intermediate in acetoacetate oxidation and that α -ketoglutarate is formed via tricarboxylic acids and not directly. Kalnitsky (58) has concluded that barium ion and magnesium ion increase citrate formation from oxaloacetate because they inhibit the utilization of citrate; cf., however, Krebs & Eggleston for an alternative interpretation.

The studies on the inhibition of respiration by fluoroacetate and fluorobutyrate have been continued by Kalnitsky & Barron (59). With animal tissue (rabbit kidney cortex homogenate) the same specificity was not found as observed previously with bacteria and yeast. With yeast, acetate oxidation and citrate synthesis were inhibited, whereas glucose oxidation was not initially inhibited, and fluorobutyrate had no effect. With kidney cortex, both fluoroacetate and fluorobutyrate gave a strong inhibition of acetate oxidation. Citrate synthesis was increased rather than decreased and glucose oxidation was inhibited immediately. The explanation of the differences is not apparent as yet, but the results cast some doubt on the specificity of fluoroacetate as an

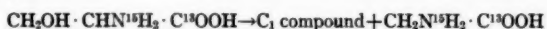
inhibitor of acetate oxidation. Black & Hutchens (60) have investigated the effect of the order of addition of fluoroacetate and acetate and have found prior addition of the inhibitor produces a long induction period but eventually the rates with and without inhibitor are the same. Barron *et al.* (61) have studied the metabolism of lung and Goldinger *et al.* (62), biological oxidation by bone marrow.

That the tricarboxylic acid cycle apparently does not operate as an important oxidative pathway in all forms of life is demonstrated by two independent lines of evidence presented by Karlsson & Barker (63), one obtained by the study of adaptive enzymes, and the other, by the isotope dilution technique. With regard to adaptive enzymes, it is assumed if a compound occurs as an intermediate in metabolism, the cell must contain the requisite enzymes. Thus it will form adaptive enzymes or have constitutive enzymes for all intermediates [Stanier (64)]. Karlsson & Barker (63) found when *Azotobacter agilis* was grown on acetate that it did not contain the enzymes necessary for oxidation of compounds occurring in the tricarboxylic acid cycle (succinate, fumarate, α -ketoglutarate). The bacteria fermented these compounds only after a latent period during which adaptation occurs. The cells grown on succinate possessed enzymes for succinate, acetate, fumarate, malate, and pyruvate but not for α -ketoglutarate. Cells grown on α -ketoglutarate were adapted to all the above compounds. The isotope dilution experiments involved addition of C^{14} -labeled succinate and normal acetate to succinate adapted cells. Although acetate was oxidized, apparently no intermediary succinate was formed from it since the isotope of the C^{14} -succinate was not diluted. These findings are taken to indicate that succinate, fumarate, and α -ketoglutarate are not intermediaries in the oxidation of acetate, and that the metabolism of acetate does not proceed via the Krebs cycle in *Azotobacter agilis*. The original article should be consulted for other tests by the dilution technique and for a more complete presentation of the argument. What the mechanism of acetate oxidation may be is of great interest since all oxidation products such as glycolic acid, oxalic acid and formic acid were found not to be attacked.

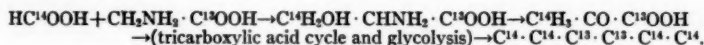
INTERCONVERSIONS TO CARBOHYDRATES VIA THE TRICARBOXYLIC ACID CYCLE

The subject has been reviewed by Wood (65, 66). Prior studies by Lorber, Lifson, Sakami & Wood of isotope distribution in the glucose derived from the liver glycogen of rats fed variously labeled lower fatty acids, and bicarbonate, have demonstrated characteristic isotope distribution patterns in the glucose [Lifson *et al.* (67)]. These findings with intact rats are in uniform agreement with the schemes of the tricarboxylic acid cycle and of anaerobic glycolysis. Recent experiments (68) of the same type using α - and α,β -labeled lactate have yielded results which are also consistent with these schemes.

The distribution of isotope in the glucose from liver glycogen may be used as an indicator of the mechanism of metabolism of other compounds. For example, it was considered that glycine might be glycogenic through reversal of the reaction discovered by Shemin (69).



C^{14} -formic acid and carboxyl labeled glycine were therefore fed to a rat and the glycogen was degraded [Sakami (70)]. The distribution was found to be a low C^{14} -concentration and a high C^{13} in the 3,4-positions of the glucose; high C^{14} and no C^{13} in the 1,6-positions; C^{14} and no C^{13} in the 2,5-positions. This distribution pattern indicated that the glycogen might be formed by the following series of reactions:



The serine from the liver proteins was therefore isolated and degraded and was found to be labeled thus, $\text{C}^{14}\text{H}_2\text{OH} \cdot \text{CHNH}_2 \cdot \text{C}^{13}\text{OOH}$, which was in conformity with predictions indicated by the glycogen result.

Ehrensverd *et al.* (71) and Winnick *et al.* (72) have found independently with Torula and liver slices respectively that labeled glycine is converted to serine, and Rittenberg (73) has pointed out that cystine with high N^{15} is formed when N^{15} glycine is fed to humans. The cysteine is believed to be formed via serine. The detailed mechanism of the conversion of formic acid and glycine to serine is of course unknown and should provide an interesting development.

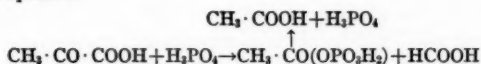
Chaikoff *et al.* (74) have fed palmitic acid labeled in the 6-position to diabetic rats and have isolated the urinary glucose. Likewise, as a control on carbon dioxide fixation, they administered radioactive bicarbonate and isolated the glucose. By comparison with the respiratory carbon dioxide there was proportionally more C^{14} in the glucose when palmitic acid was fed than when the $NaHC^{14}O_3$ was administered. This indicated that there is a mechanism for conversion of palmitic acid to glucose other than by carbon dioxide fixation. No degradation of the sugar was done, but these results will be awaited with interest. By beta oxidation it is to be expected that a methyl-labeled acetyl group would be formed and this should give rise to a sugar labeled in all positions but with equal and highest concentration in the 1,2,5,6-positions.

It has now been shown by Gurin *et al.* (75) in phlorizinized rats that a compound which is glucogenic and antiketogenic nevertheless may contribute part of its carbon to ketone bodies. When α,β -labeled lactate was fed 28 per cent of the administered isotope appeared in the urinary glucose whereas 10.2 per cent appeared in the ketone bodies and 7.3 and 22.4 per cent in the fat of liver and muscle respectively. Estimation of the extra urinary glucose by Lusk's procedure indicated that an equivalent of 70 per cent of the lactate fed was excreted as glucose whereas only 28 per cent of the isotope was excreted as glucose. Viewed in terms of dynamic equilibria, the metabolic pool, and interconversion mechanisms, it is not surprising that carbon of a glucogenic compound would appear in fats. The problem of how the balance of conversion in the metabolic pool is altered so as to shift the relative proportion of ketone and glucose production is, of course, an unanswered puzzle. All four carbons of the excreted acetoacetic acid were found to be radioactive and the specific activity was found to be higher than the body fat. Carboxyl-labeled alanine was likewise used in these studies. In this case 1 to 5 per cent of the administered isotope was recovered in the glucose.

THE ROLE OF ACETYL PHOSPHATE IN SYNTHESIS

Acetyl phosphate has long been suspected of taking part in syntheses involving two carbon compounds but the proof has thus far been lacking with a well-defined system. The best evidence has been with a cell-free enzyme preparation from *E. coli* with which it was shown that a cleavage of pyruvate occurs with formation of

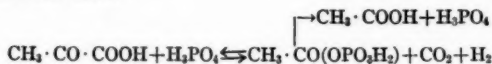
acetate and formate and a compound having the properties of acetyl phosphate.



By adding labeled formate and normal pyruvate to the system it was shown that formate was fixed in the residual pyruvate (76). The assumption was therefore made that the reaction was reversible and involved acetyl phosphate. However, Strecker *et al.* (77) have recently tested the system using the *E. coli* enzyme, together with $\text{CH}_3 \cdot \text{C}^{13}\text{O}(\text{OPO}_3\text{H}_2)$, HC^{14}OOH , and nonisotopic pyruvate and have found that C^{14} -formate enters the carboxyl group of the pyruvate but the synthetic C^{13} -acetyl phosphate does not accompany the formate. The results show that the monoacetyl phosphate as synthesized is not a direct intermediate of the reaction by which formate was fixed in pyruvate.

Kaplan & Lipmann (78) at the same time made the very interesting discovery that a compound which contains a labile phosphate but differs from the synthetically prepared acetyl phosphate is formed from acetate and ATP by an extract from *E. coli*. The difference was detected by measuring the rates of hydrolysis using a muscle enzyme. The product has now been partially purified by Kaplan & Lipmann (79), and it has been found that by short exposure to pH 1.5, even at room temperature, the product changes so that it is no longer distinguishable from the synthetic acetyl phosphate. Even more important is the fact that the natural product gives an almost quantitative yield of pyruvate when it is fermented with dried *E. coli* and formate. On the other hand synthetic monoacetyl phosphate gives no synthesis of pyruvate if substituted for the natural product in the above reaction. The results seem to indicate that the product from the acetate and the ATP reaction may be the long awaited active two-carbon intermediate which takes part in synthetic reactions. Developments will be awaited with interest.

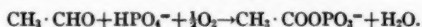
A reaction which is very similar to the phosphoclastic reaction of *E. coli* is produced by an extract from *Clostridium butylicum*. The difference is that carbon dioxide and hydrogen are formed rather than formate.



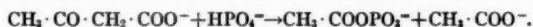
Koepsell *et al.* (80) demonstrated that a labile phosphate is formed in the reaction and some evidence of its reversibility was presented by Lipmann & Tuttle (81). Wilson *et al.* (82) have now investigated the problem with C^{13} -labeled compounds. They have found by incubation of the enzyme with pyruvate, $NaHC^{13}O_3$, and hydrogen that the carboxyl group of the pyruvate exchanges with bicarbonate and the C^{13} concentration of the carboxyl group and residual $NaHC^{13}O_3$ become practically equivalent. This result is comparable to that obtained with the *E. coli* juice and C^{13} formate (76). It is to be noted, however, that in the *C. butylicum* reaction formate is not an intermediate. This was shown by Wilson *et al.* by tests with $HC^{13}OOH$ in which no isotope was fixed into the pyruvate. When $CH_3 \cdot C^{13}OOH$ and ATP or acetyl phosphate, or adenylic acid and $CH_3 \cdot C^{13}OOH$ were tested, it was found that C^{13} was introduced into the carbonyl group of the pyruvate. Without addition of ATP or acetyl phosphate the amount of C^{13} incorporated from $CH_3C^{13}OOH$ was somewhat less. However, the reviewers find the results from the $CH_3C^{13}OOH$ experiments very puzzling because there is a major discrepancy between the observed C^{13} in the residual acetate and that which is to be expected from the reaction under study. For example, 1.1 mM of acetate with 4.65 per cent excess C^{13} was used with 2.0 mM of pyruvate. The maximum dilution of the acetate that would be expected would be one acetate from each pyruvate or a dilution of 1.1 to 3.1 which gives a calculated value of 1.65 per cent excess C^{13} for the residual acetate whereas a value of 0.11 was the concentration reported. The concentration of C^{13} in the $CH_3 \cdot CO$ -portion of the pyruvate was between 0.04 and 0.12 per cent excess C^{13} . It appears that either there was an error in the analysis of C^{13} in the residual acetate or there are major pathways of acetate metabolism in the system studied other than those indicated in the above equation. It is apparent that compared with the calculated value of 1.65 the exchange of acetate in pyruvate was not very large. On this basis the results are comparable to those of Utter *et al.* (76) with *E. coli* juice. They found that acetate entered the pyruvate in about one-twentieth the amount as did formate.

New evidence of the importance of acetyl phosphate has been found by Stadtman & Barker (83) who reported two new reactions with *Clostridium kluyveri* in which acetyl phosphate is formed.

One is the oxidation of ethanol or acetaldehyde with uptake of inorganic phosphate to form acetyl phosphate.



The other is a phosphoclastic split of acetoacetic acid.

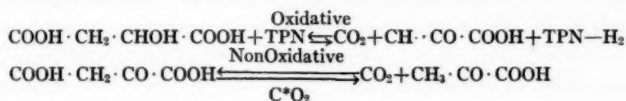


The bacterium, *C. kluyveri*, is of further interest because it has provided an admirable tool for study of fatty acid synthesis from ethanol and acetate by Bornstein & Barker (84). They have demonstrated the synthesis of butyrate and caproate from ethanol and acetate, and it appears probable that this synthesis of fatty acids may take place with acetyl phosphate as a component. This is especially indicated by the experiments [Soodak & Lipmann (85)] in which acetoacetate was synthesized with a liver enzyme requiring ATP, coenzyme A and acetate.

CARBON DIOXIDE FIXATION

The complete reports (86 to 92) are now available on the excellent experiments by Ochoa and his co-workers in which they studied carbon dioxide fixation indirectly by spectrophotometric means. Their principal findings have been reviewed by Vennesland in the 1948 *Annual Review of Biochemistry* [cf. also Ochoa (93)].

The results on carbon dioxide fixation are somewhat confusing at present, probably because of differences in the methods of measurement. The spectrophotometric method of necessity entails a biological oxidation, since the course of the reaction is followed by determining the change in the amount of oxidized or reduced triphosphopyridine nucleotide (TPN). On the other hand the tracer studies with oxaloacetate have been made by addition of labeled carbon dioxide to systems where no known oxidation or reduction is occurring and the results are measured by determining the incorporation of isotope in the oxaloacetate (94). For example



As determined by these two methods the requirements for fixation of CO_2 in C_4 acids are different. The nonoxidative fixation of

carbon dioxide is stimulated by ATP and is not influenced by TPN or DPN (65, 95, 96), whereas the oxidative fixation is stimulated by TPN and does not require ATP or phosphate. As proof of the latter point Ochoa *et al.* (91) by repeated fractionation of the malate-pyruvate enzyme reduced the phosphate concentration to 10^{-6} M and the rate of reduction of TPN by malate was not affected either by addition of inorganic phosphate or ATP. Thus far it has been impossible to separate the oxidative and non-oxidative enzymes (91). In fact the two activities have paralleled each other throughout the purification, i.e., the ratio of the rate of nonoxidative decarboxylation of oxaloacetate and oxidative dehydrogenation of malate was constant. It is possible that a single enzyme is involved with a dual function and with different cofactors for these functions. Thus ATP might be required to activate an intermediate nonoxidatively whereas in the oxidative conversion the intermediate would be formed without ATP.

A malate-pyruvate enzyme has recently been obtained from bacteria which is DPN specific (92) instead of TPN specific.

The status of the fixation of carbon dioxide in tricarboxylic acids (86, 87, 88) is comparable in many respects to the fixation of carbon dioxide in C_4 -dicarboxylic acids. Isocitrate dehydrogenase has been found to be accompanied by oxalosuccinate carboxylase and separation has not been accomplished. It thus is not certain what the interrelation of the decarboxylase and dehydrogenase may be in the fixation. There is no direct evidence of a nonoxidative fixation of carbon dioxide in oxalosuccinate, i.e., fixation with a system consisting of oxalosuccinate, labeled carbon dioxide, the enzyme and perhaps ATP. Vennesland *et al.* (97) have shown that ATP stimulates fixation of isotopic carbon dioxide by the parsely root enzyme which suggests that there may be an ATP mechanism for fixation in oxalosuccinate similar to that of the nonoxidative fixation in oxaloacetate. This is in contrast to Ochoa's results using spectrophotometric methods and the enzyme from heart where no effect of ATP was found.

Kornberg *et al.* (89) have presented spectrometric evidence that manganous and aluminum ions form complexes with oxaloacetate and oxalosuccinate and that the formation of the complex is stimulated by the respective carboxylases for these compounds.

Schubert & Armstrong (98) have turned up an interesting observation. They have found that $C^{14}O_2$ is fixed in fatty acids and

that the carboxyl of the saturated fatty acids has approximately twice the specific activity of that of the total carbon of the acid, indicating that the isotope is in the odd carbons of the saturated fatty acids. According to current theories such a fatty acid would be built up from carboxyl-labeled acetyl groups, thus requiring that carbon dioxide carbon be incorporated into the carboxyl of acetic acid or acetyl groups. The mechanism whereby this fixation could occur is not known and heretofore there has been no indication of such fixation in animals. A possible explanation is the formation of acetate by central cleavage of succinate; but here again evidence is lacking for this reaction, especially with animal tissue. Armstrong *et al.* (99) have also studied in long term experiments with rats, the uptake of $C^{14}O_2$ in glycogen of liver and muscle.

Whereas there is ample evidence that biotin has some function in carbon dioxide fixation (cf. last year's *Annual Review of Biochemistry*) there is no evidence as to how it functions or that it is part of the enzyme for fixation.

There has been considerable speculation that the mechanisms of photosynthetic fixation of carbon dioxide may resemble in certain aspects the heterotrophic fixation of CO_2 . While it is much too early to consider the identity or the mechanisms of the initial or primary fixation of carbon dioxide in photosynthesis there are indications that plants may utilize some reactions similar to those of animals. The greatest concentration of isotopic carbon dioxide appears first in the 3,4-positions of the sugar and the concentration is next highest in the 2,5-positions and lowest in the 1,6-positions. This has been shown by Aronoff *et al.* (100) with barley roots and by Wood & Burr with bean plants and sugar cane (101). The occurrence of isotope in highest concentration in the 3,4-positions is in striking similarity to the results with carbon dioxide fixation in rat liver glycogen (102). The more recent results of Calvin & Benson (103) and of Stepka *et al.* (104) show that a 30-second exposure of algae to light leads to a greater fixation in the sugars than does the dark fixation, and that in these short exposures 75 to 90 per cent of the fixed carbon dioxide in the glucose is in the 3,4-positions. A fraction believed to contain triose contained 70 per cent of the total fixed carbon dioxide. Aspartic acid and alanine contained a large part of the radioactivity of the amino acid fraction. Glutamic acid had no isotope which seems to indicate that α -ketoglutarate and the tricarboxylic acid cycle are not on the main path of photo-

synthesis. As a working hypothesis (103) it has been proposed that carbon dioxide combines with acetate to yield pyruvate. Pyruvate then combines with carbon dioxide to give oxaloacetate, the oxaloacetate is reduced to succinate and succinate is split to two acetate molecules which on combination with carbon dioxide yield "two" pyruvate molecules.

DIABETES, INSULIN, AND RELATED TOPICS

These areas of carbohydrate metabolism have received much attention. A number of studies on isolated tissues and enzyme systems have appeared. Reid, Smith & Young (105) have reported confirmation (no data are given) of the finding, originally made by Price, Cori & Colowick (106), that a factor in anterior pituitary extracts inhibits hexokinase activity in *in vitro* preparations of various tissues, and that the inhibition is reversed by insulin. Reid *et al.* have found no correspondence between the ability of a given extract to produce an insulin-reversible inhibition of the hexokinase reaction *in vitro*, and its diabetogenic activity *in vivo*. They report, however, that when preparations that exhibit only diabetogenic activity are administered to rats, the muscle hexokinase from these animals shows increased activity on the *in vitro* addition of insulin. The authors speculate that the diabetogenic factor may be the hexokinase inhibitor, but that it loses its activity on storage and regains it *in vivo*.

Bornstein & Nelson (107), Krah1 & Park (108), and Perlmutter & Greep (109) have compared the effect of insulin on the glucose uptake and/or glycogen deposition in the isolated diaphragm of normal and hypophysectomized rats. Where comparable experiments have been done, results are basically in agreement, and indicate that the diaphragms from both the normal and hypophysectomized animals show enhanced glucose uptake and glycogen deposition in response to insulin, while in the absence of insulin tissue from the operated animals exhibits greater activity than that from intact animals.

Assuming that the rate of glucose uptake is a measure of hexokinase activity in the isolated diaphragm (a premise accepted by several of the authors cited above), supernormal glucose uptake by diaphragm from hypophysectomized rats is in accord with a pituitary inhibition of hexokinase, while the fact that insulin produced an effect in tissue from operated animals is not in harmony

with the thesis that insulin affects hexokinase solely by releasing the enzyme from inhibition by a pituitary factor. The following alternative postulates have been advanced to explain the results: (a) a nonpituitary, insulin-reversible inhibition of hexokinase; (b) a direct effect, in the intact cell, of insulin on the enzyme (independent of pituitary inhibition); or (c) an insulin-sensitive enzyme system peculiar to the diaphragm. The possibilities remain that differences noted in glucose uptake by the isolated diaphragms were not (or not entirely) manifestations of altered hexokinase activity, that ATP concentration may have been a (or the) limiting factor, and that insulin may have promoted glucose uptake in the absence of pituitary inhibition by stimulating ATP formation via an effect on an enzyme system involved in energy-yielding reactions. Support for this possibility is gained from the observations of Stadie, Haugaard & Perlmuter (110), who have confirmed earlier observations by others of a stimulating effect of insulin on pyruvate utilization by pigeon breast muscle preparations, and from the report of Bloch & Kramer (111) who have shown an increased incorporation of acetate carbon into higher fatty acids on the addition of insulin to rat liver slices incubated with pyruvate and isotopic acetate.

Zwilling (112) has reported a hypoglycemia induced by insulin in the chick embryo presumably before the appearance of anterior pituitary and adrenal cortical secretions, indicating that in the chick embryo also insulin is able to produce an effect independent of the pituitary.

Tuerkischer & Wertheimer (113) have studied the effect of insulin on glycogen deposition in diaphragms from normal and alloxan-diabetic rats, suspended in various media. In saline media glycogenesis was enhanced by insulin in both the normal and diabetic tissue. When, however, normal and diabetic sera were used, striking differences appeared. In normal serum, normal diaphragm failed to show insulin stimulation while diabetic diaphragm responded. In diabetic serum, glycogenesis was depressed in both normal and abnormal tissue, the latter responding to insulin markedly. These observations, together with the finding that saline resembling extracellular fluid inhibits glycogenesis in the diaphragm while supporting it (as shown previously by Hastings & Buchanan (114)) in liver slices, re-emphasize the importance of the suspending medium in the *in vitro* study of such

complex systems as the diaphragm and liver slice. The difficulties in interpreting results obtained with such systems is further underscored by the cytochemical studies of Deane *et al.* (115) who found glycogenesis occurring at the periphery of liver slices concurrently with glycogenolysis at the center, and by the finding of Stadie *et al.* (116) that glycogenesis proceeds optimally in the rat heart slice when the suspending medium is virtually devoid of ions!

Broh-Kahn & Mirsky (117) have found no effect of insulin on phosphorylase, phosphoglucomutase, glucose-6-phosphatase, and PR (a phosphorylase-inactivating factor), studied in cell-free extracts from livers of normal and alloxanized rats. It is unclear whether or not the enzyme was established as the rate-limiting factor in these experiments.

A number of papers have appeared dealing with the problems of diabetes and insulin action in intact normal and diabetic animals. Zilversmit *et al.* (118) have studied the rate of conversion of C^{14} -labeled glucose to respiratory carbon dioxide in normal and alloxan-diabetic rats and have calculated the rate of glucose oxidation from an average value for the specific activity of the plasma glucose over the experimental period. They calculated that the diabetic rat oxidizes glucose at a normal rate. This is in apparent conflict with the report of Villee, Sinex & Solomon (119) who studied glucose oxidation in the isolated diaphragm, using C^{14} -labeled glucose, and found glucose oxidation depressed in the diabetic muscle. There is no indication, however, that they studied the rate of glucose oxidation in the diabetic diaphragm at diabetic glucose concentrations. Under such circumstances agreement with the findings of Zilversmit *et al.* might have resulted. It may be superfluous to point out that a normal rate of glucose oxidation at the hyperglycemic levels in diabetes cannot be taken to mean that the mechanisms involved are functioning normally.

Tuerkischer & Wertheimer (120) have reinvestigated the problem of glycogen deposition in the livers of alloxan-diabetic rats, and have confirmed their earlier findings showing that liver glycogen levels of the fed diabetic rat without acidosis compare favorably with values for pair-fed normal controls, and that the fasting diabetic animal actually has a higher liver glycogen than the fasting normal. In addition, the fasting diabetic animal deposited more liver glycogen in response to glucose feeding than did the normal control. In contrast, the diabetic animal with acidosis showed

consistently lower liver glycogen values than the corresponding normal controls, and failed to respond to glucose administration. The authors suggest that the difference between the diabetic animal with and without acidosis may be accounted for by the nonacidotic animal being only partially diabetic, i.e., still possessing some functional islet tissue. It is difficult at present to account for the differences between the normal and nonacidotic diabetic rats on this basis alone. The findings reported in this latter group of rats are not in accord with established notions regarding the behavior of liver glycogen in diabetes. Either the results are a consequence of special circumstances and are applicable only to the alloxan-diabetic rat without acidosis, or established notions need re-examination.

Nath & Brahmachari (121) have continued their studies on the possible diabetogenic role of ketone bodies. After prolonged injection into the guinea pig, a reduced insulin content was found in the pancreas. Tidwell & Axelrod (122) using rats have failed to find the reduced carbohydrate tolerance following acetoacetate injection similar to that described by Nath & Brahmachari in prior experiments in rabbits. However, after a single large injection of acetoacetate, they noted a hypoglycemia, possibly due to a stimulation of insulin secretion. The problem is one of obvious importance and should be studied further.

Conn, Lawrence & Wheeler (123) administered adrenocorticotrophic hormone to normal human adults and observed hyperglycemia, lowered glucose tolerance and negative nitrogen balance. A close temporal relationship was observed between loss of glucose tolerance and increased urinary excretion of uric acid. In one subject in whom the determination was made, the reduced glutathione in the blood was low. The authors suggest the possibility of an alloxan-like intermediary metabolite as the agent responsible for the diabetic response. Space does not permit elaboration of the rationale involved, but the thesis, proposed earlier by Lazarow (124), is an attractive one and deserves further attention. Griffiths (125), working along the same line, has produced transient hyperglycemia and glycosuria by injecting uric acid into rabbits placed on a methionine and cystine-deficient diet.

Ingle & Prestrud (126) have attempted a re-evaluation of the ameliorating effect of adrenalectomy on diabetes, using the force-fed, partially depancreatized rat. The improvement in the

diabetes following removal of the adrenals has generally been assigned to a decreased gluconeogenesis from protein. Force-feeding was employed by Ingle & Prestrud to obviate the effects of diminished food intake following adrenalectomy. After establishing a diabetic state the adrenals were removed and the animals maintained without hormone therapy for 14 days. During this period glucose disappeared from the urine and urinary nitrogen fell to pre-diabetic levels. The decrease in urinary nitrogen could account for only a small part of the decrease in urinary glucose, and it was therefore suggested that a large part of the effect of adrenalectomy had resulted either from a decreased gluconeogenesis from fat, and/or an increased utilization of carbohydrate. In the absence of an established chemical pathway for the net formation of carbohydrate from fat in mammalian tissue, the question of gluconeogenesis from fat must remain open. The role of gluconeogenesis from proetin would also appear to remain largely unsettled since it is unclear how one is to assign values of glucose equivalence to the urinary nitrogen. Thus, although the decrement in urinary nitrogen following adrenalectomy could account for only a small fraction of the glucose that disappeared from the urine over the same period, it is possible that the protein represented by the remaining urinary nitrogen was transformed to glucose to a lesser extent than an equivalent amount of protein prior to adrenalectomy. It appears, however, that enhanced glucose utilization may be a real factor in the improvement in the diabetic state following adrenalectomy. Ingle & Nezamis (127) have reported an increased glucose tolerance in adrenalectomized eviscerated rats, as compared with eviscerated controls, and Villee *et al.* (119) have found an increased rate of glucose oxidation in the isolated diaphragm of the adrenalectomized rat. Increased glucose uptake by the isolated diaphragm of the diabetic adrenalectomized rat compared to that of the diabetic rat has been observed previously [Krahl & Cori (128)], and Reiss & Rees (129) have reported increased glucose disappearance in rat brain homogenates from adrenalectomized and hypophysectomized animals. Adrenalectomy may act by removing a factor that potentiates a pituitary inhibition of the hexokinase reaction, as suggested by the findings of Colowick *et al.* (130) using a cell-free enzyme preparation. The results of Conn *et al.* (123) indicate a decreased carbohydrate utilization in humans given adrenocorticotrophic hormone.

The role of phosphatases of liver and kidney in relation to carbohydrate metabolism and diabetes has received further attention. Drabkin & Marsh (131) have observed an increase in both acid and alkaline phosphatase activity of liver in established alloxan diabetes in the rat. Kochakian & Bartlett (132) have administered various adrenal cortical preparations, thyroxine, and epinephrine, separately to rats, and have studied the effects on liver glycogen and alkaline phosphatase. Changes in glycogen and enzyme activity were apparently unrelated. Bunting (133) observed a decrease in kidney alkaline phosphatase in alloxan diabetes, and no change during hypoglycemia induced by insulin or during hyperglycemia induced by glucose injection. This last is in contrast to a prior report by Marsh & Drabkin (134) of an increase in both acid and alkaline kidney phosphatase in response to alimentary hyperglycemia in rats. The role of phosphatases in the development of the diabetic syndrome appears to be unsettled.

The possibility of a second carbohydrate-active pancreatic hormone is under active investigation. It has been known for some time that a hyperglycemic factor is present in most commercial insulins. That a blood sugar-raising factor is produced by the pancreas has been suggested earlier on the basis of differences between the alloxan-diabetic dog and the depancreatized dog (135), and certain clinical observations. Zimmerman & Donovan (136) have studied the known hyperglycemic effect of insulin in preparations in which the insulin itself has been inactivated, and conclude that the hyperglycemic factor operates independently and not as a direct antagonist of insulin. Heard *et al.* (137) prepared an insulin-free pancreatic extract which produced hyperglycemia when injected into rats, and an increased liberation of glucose into the medium when incubated with rat liver slices. Sutherland & Cori (138) observed an increased conversion of glycogen to glucose in liver slices of well-fed rats and rabbits on addition of amorphous or crystalline insulins. The glycogenolytic activity remained after inactivation of the insulin, and was found to be nondialyzable, destroyed by trypsin, and removed by trichloroacetic acid. It apparently required an intact cell structure for its effect. Other proteins tested did not enhance glycogenolysis. The liver slices put out large amounts of glucose when glucose-1-phosphate was added to the medium, indicating that phosphoglucomutase and

glucose-6-phosphatase activities were not limiting, and suggesting phosphorylase as the site of action of the glycogenolytic factor, though not establishing it with finality. Sutherland & de Duve (139) have studied the distribution of the factor and have found increased amounts per unit weight of tissue after ligation of the pancreatic ducts and degeneration of acinar tissue. Normal amounts could be extracted from the pancreas of an alloxan-treated animal, leading to the inference that the α cells of the islets of Langerhans are the source of the glycogenolytic factor. General recognition of the factor is of practical importance in the study of insulin action. Establishment of the factor as a pancreatic hormone will represent a significant advance in the fields of carbohydrate metabolism and diabetes and further developments are eagerly awaited.

Space permits little more than the listing of a number of other interesting papers in the field of diabetes and insulin action. The mechanism of the blood sugar changes induced by alloxan in the period prior to the establishment of diabetes has been investigated by Griffiths (140, 141), and Banerjee & Bhattacharya (142). Davis (143) has described a parallelism between blood glucose and total blood iodine in the alloxan-diabetic dog. Beatty (144) has studied the hemorrhage-induced hyperglycemic response in the normal and alloxanized dog. The influence of pantothenic acid deficiency, thiamine deficiency, and high fat diet on the course of alloxan diabetes in the rat have been investigated by Irwin & Ralli (145), Janes & Brady (146), and Janes & Prosser (147), respectively. Waisbren (148) has studied the conditions necessary for the successful induction of alloxan diabetes in mice, while Manhoff & DeLoach (149) have reported similar study in the dog. West & Highet (150) have confirmed the refractoriness of the guinea pig to alloxan. Houssay & Martinez (151) have investigated the mechanism by which clamping of the renal pedicles protects the dog against alloxan. The subject of alloxan diabetes has been reviewed by Lukens (152).

Mirsky *et al.* (153) have measured urinary insulin excretion in normal man and report that large injections of insulin cause little increase in output. Broh-Kahn & Mirsky (154) have described a heat-labile factor from liver which inactivates insulin, and is presumed not to be one of the known proteolytic enzymes. Kety *et al.* (155), measuring cerebral blood flow by the nitrous oxide method, have investigated cerebral oxygen and glucose uptake in

humans during insulin coma and have found a reduction in both. Glucose consumption fell more than did oxygen uptake. Somogyi (156) has noted a decreased glucose tolerance during insulin-induced hypoglycemia in normal adults, presumably due to an insulin-antagonistic compensatory mechanism. Bone & Reid (157) have noted a fall in blood amino nitrogen following administration of insulin to the fasted cat. This could be prevented by giving glucose with the insulin. Slater *et al.* (158) have followed the course of development of insulin hypersensitivity in the hypophysectomized dog and suggest a relationship to the secondary atrophy of the adrenals and thyroid. Chase *et al.* (159) have demonstrated extreme insulin tolerance in an inbred strain of mice. This is a finding of some practical importance in connection with insulin assay, and may provide an approach to some aspects of the problem of insulin action.

The problem of insulin action and related areas in carbohydrate metabolism has been reviewed by Young (160). This paper, written before the more recent work on the hyperglycemic factor in insulin had appeared, conveys the erroneous impression that insulin itself is responsible for effects produced by the factor.

MISCELLANEOUS

Under this heading are listed a number of papers dealing with physiological, nutritional, hormonal, and other aspects of carbohydrate metabolism.

Reinecke and co-workers (161, 162) have continued their studies on the kidney as a source of blood glucose, using the arteriovenous difference method. Ingle (163) and Ingle & Nezamis (164) have determined blood glucose levels in the eviscerate rat under various conditions, in the presence and absence of the kidneys, and have generally found higher values in the presence of the kidneys. The physiological role of the kidney in blood sugar regulation remains to be determined.

Attempts to evaluate neural mechanisms of blood sugar control have been made by Lackey (165), Weiland, Broh-Kahn & Mirsky (166), and Gregory, Bennett & May (167). Lackey and Weiland *et al.* have failed to confirm prior reports of hypoglycemia following intrathecal glucose injection. Gregory *et al.* applied a faradic current to the cervical vagus in the dog and found a hypoglycemic response in one out of nine animals. This was interpreted as evi-

dence against a vagal role in insulin secretion, but it is by no means conclusive.

Myers (168), employing catheterization of the hepatic veins in human subjects, has presented evidence indicating that extrahepatic tissues are responsible for the specific dynamic action of intravenously administered glucose. Cunningham, Barnes & Todd (169) have found liver glycogen relatively high in the 24-hour fasted rat on a prior diet high in protein or glycine. This may result from the delayed glycogenic response known to follow glycine feeding (170). The reason for this delay is obscure but may be due in part to the time required to convert appreciable amounts of glycine to glycogen precursors via serine, which appears to lie on the path linking glycine and glycogen (70).

Other subjects investigated include: the relationship of prior fat feeding to carbohydrate intake in the rat and the glucose uptake and glycogen deposition by the isolated diaphragm of rats on high fat and high carbohydrate diets [Lundbaek & Stevenson (171, 172)]; the influence of fructose on glucose utilization by the isolated rat diaphragm [Corkill & Nelson (173)]; the effect of anoxia on glucose tolerance in dogs [Stickney, Northup & Van Lier (174), Van Lier *et al.* (175), and Kelley & McDonald (176)]; the effects of hyper- and hypoglycemia on the arteriovenous glucose differences in normal human subjects [Somogyi (177, 178)]; epinephrine hyperglycemia in hypophysectomized dogs [de Bodo *et al.* (179)]; the glucose utilization of muscle preparations from epinephrine-injected rats [Cohen & Needham (180)]; the influence of adrenal cortical hormones on glycogen synthesis in the isolated rat diaphragm and liver slice [Verzár & Wenner (181, 182, 183), and Chiu & Needham (184)]; the assay of carbohydrate-active adrenal compounds in the adrenal vein blood of the dog [Paschkis *et al.* (185)]; a water soluble factor from the adrenal cortex, reported to have striking effects on the synthesis of glycogen and creatine phosphate in various tissues [Medvedeva (186)]; the influence of male hormone on the secretion of fructose and citric acid by transplanted accessory sex glands of the male rat [Mann, Lutwak-Mann & Price (187)]; galactose utilization in weanling and adult rats, and rats with hepatoma [Derse, Elvehjem & Hart (188)]; galactose and glucose tolerance in a child intolerant to galactose [Greenman & Rathbun (189)]; deposition of liver glycogen in normal and sarcoma-bearing mice [Young *et al.* (190)]; some

effects of bacterial endotoxins on carbohydrate metabolism in rats and rabbits [Kun & Miller (191) and Kun (192, 193)]; and the effect of insulin on phosphate turnover in traumatic shock [Goranson, Hamilton & Haist (194)].

Space permits only the briefest comment on a few of the foregoing papers. It is interesting that the weanling rats in which Derse, Elvehjem & Hart (188) studied galactose utilization developed cataracts on a skim milk diet, and that Greenman & Rathbun (189) have described a similar finding in their galactose-intolerant infant. Goranson, Hamilton & Haist (194) have reported an increased incorporation of P^{32} in muscle phosphocreatine and ATP following insulin administration in rats. This could be the result of a higher specific activity of intracellular inorganic phosphate due to an increased movement of plasma glucose and phosphate into cells following insulin administration, rather than or in addition to an increased turnover rate, as the authors have concluded. Whether or not this factor has been taken into account is not clear from the report.

METHODS

Among the many new or modified methods reported, the following are listed: a photometric method for free pentoses in tissues [Roe & Rice (195)]; a photometric micromethod for methyl-pentoses [Dische & Shettles (196)]; an anthrone reagent applicable to the determination of carbohydrates in general [Morris (197)]; a cytochemical method for the demonstration of polysaccharide [Hotchkiss (198)]; an enzymatic method specific for glucose [Keilin & Hartree (199)]; a colorimetric method for hexoses using carbazole [Holzman, MacAllister & Niemann (200)]; new developments and applications of filter paper chromatography in the separation of sugars [Forsyth (201), Forsyth & Webley (202), and Partridge & Westall (203)]; a powdered cellulose column for the separation of sugars and their methylated derivatives [Hough, Jones & Wadman (204)]; a color reaction for galacturonic acid [Dische (205)]; and the biosynthesis of C^{14} -labeled starch and sugars [Putman *et al.* (206)].

REVIEWS

A number of reviews and monographs have appeared which bear wholly or in part on the material dealt with in this review.

Some of these are listed briefly: glycolysis and phosphorylation [Meyerhof (207)], various aspects of the problems of insulin action and diabetes [Laszt (208), Burger (209), Lukens (152), and Young (160)], biosynthesis of polysaccharides [Hehre (210)], structure of glycogens [Bell (211)], an introduction to carbohydrate biochemistry [Bell (212)], uses of radioisotopes in biology [Sacks (213)], and fatty acid metabolism [Breusch (214) and Leloir (215)].

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MINERAL METABOLISM¹ (FLUORINE AND OTHER TRACE ELEMENTS)

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Fluorine is of particular interest at this time because of its association with conspicuous activity in the field of preventive dentistry (1). Direct fluorination of public water supplies is being investigated in at least 12 communities in the United States and Canada, as a mass caries control measure for large population groups (2, 3), and a very extensive program relative to the topical application of fluoride to tooth surfaces of children is in progress (4). A daily supplement of fluoride to children's diets during formative tooth life is also a suggested caries control measure (5) where fluoride drinking water is not available. At the same time, the indiscriminate use of fluoride must be seriously condemned because of the toxic potentialities of continued and excessive fluoride ingestion (6, 7).

Trace mineral elements in general became more closely identified with enzyme and vitamin activity by the discovery that cobalt is an essential part of the new antianemia vitamin B₁₂ (8). Previously, zinc was demonstrated in purified carbonic anhydrase (9). Radioactive minerals have been useful in identifying the body organs and secretions most active in mineral exchange, but no new evidence would seem to permit additions to the current list of physiologically essential mineral elements.

This review is limited by restrictions of space; in addition to fluorine, it deals only with manganese, cobalt, zinc, and boron. A number of other reviews of mineral elements have appeared in recent years (10 to 17).

FLUORINE

Fluorine and dental caries.—A relation of fluoride domestic waters to dental caries was first suggested by epidemiological studies which demonstrated that excessive fluoride in drinking water is the cause of endemic mottled enamel (6). Results of subsequent epidemiological surveys (1) now permit conclusive as-

¹ This review covers the period from January, 1943 to September, 1948.

sociation of domestic waters of optimal fluoride content (1.0 to 1.5 p.p.m. fluorine) with a reduced dental caries experience in 12 to 14 year old children. Dean and his associates (18) found the intensity of caries attack in school populations (age 12 to 14 years) in eight suburban Chicago communities was inversely correlated with 0.5 to 1.8 p.p.m. fluorine in drinking water. A maximum caries reduction of about 60 per cent resulted from continuous exposure to 1.5 to 1.8 p.p.m. fluorine in the drinking water. Other epidemiological studies support the fluorine-dental caries relationship (19 to 22).

Whereas caries inhibition has been most effectively demonstrated for 12 to 14 year old children continuously exposed to fluoride waters, any assurance of inhibitory effects in older individuals must await further detailed studies. It has been observed, however, that young adult men whose exposure to fluoride waters followed their formative tooth life (23, 24) had a reduced dental caries incidence.

Weaver extended his observations in England (25) and reported on a group of 100 women, aged 20 years to "40 and over," that there was a dental advantage due to continuous exposure to 1.4 p.p.m. fluorine in drinking water, "of about five years on the average," i.e., an age was eventually reached when these adults' dental caries experience was the same with and without fluorine exposure (26). Weaver suggested that "fluorine is a caries postponing, rather than a caries preventing factor."

Contrary to Weaver is the conclusion of McKay (27), i.e., that teeth having acquired a resistance to decay through exposure to fluoride waters, retain this resistance "well into adult life." These conflicting reports (26, 27) are both based on numbers of adults too small to permit conclusive statements. They emphasize, however, the many unresolved factors connected with the mechanism as well as the duration of the fluoride anticaries effects and suggest a conservative attitude toward the role of fluorine in preventive dentistry.

Experimental results have consistently supported the epidemiological evidence of a human caries-fluorine relationship, i.e., (a) induced experimental caries in small animals is fluoride-inhibited (28 to 32); (b) dental tissues contain fluorine which may be classified as primary fluorine, acquired during formative tooth life (33), secondary fluorine acquired after tooth eruption (34, 35,

36), and adsorbed secondary enamel fluorine (37, 38, 39) acquired possibly by local oral enamel surface adsorption; (c) reduced acid solubility (28, 40 to 45), and surface changes in the enamel (46, 47) and dentin (48) are attributed to fluorine reactions on dental tissues *in vitro*; (d) fluoride may affect oral bacterial activity (49, 50) and exert antienzymatic effects possibly associated with dental caries etiology; and (e) the ameloblasts (enamel forming cells) are extremely sensitive to fluorine (51).

The fluorine content of dentin and enamel is obviously of extreme interest in explaining fluorine-dental caries relations, and limited analytical data reported by Armstrong & Brekhus (52) have supported the view that enamel of carious teeth contains less fluorine than enamel of sound teeth. However, other data (53) and a more extensive study by McClure (54) refute this idea as applied to individual teeth. Dentin and enamel of several hundred sound and carious teeth, which showed no evidence of fluorosis, and which were obtained from nearly 100 individuals, did not differ in fluorine content with any regard to their carious or noncarious histories (54). Enamel of sound and carious teeth contained 0.0102 ± 0.004 per cent and 0.0098 ± 0.003 per cent fluorine, respectively, and dentin from sound teeth contained 0.0241 ± 0.0010 per cent fluorine and from carious teeth 0.0225 ± 0.0007 per cent fluorine. Teeth of different types (incisors, molars, etc.) did not differ in fluorine content. It was suggested (54) that an increase of fluorine in an entire dentition may account for an overall reduction in dental caries, but that individual carious teeth can not be expected to contain consistently less fluorine in the enamel (or dentin) than do sound teeth.

Evidence that the fluorine content per se is associated with and may influence the physical and biochemical properties of teeth *in vivo* may be cited as follows. Excess fluorine is found in dentin and enamel of hypoplastic (mottled enamel) human teeth (55, 56, 57) and fluorine content correlates directly with the severity of the typical rat-incisor hypoplasia produced by dietary fluorine (58). Caries-preventive effects also are now being reported in the majority of studies of topical applications of fluoride to tooth surfaces (59 to 69). While the analytical procedures have failed to demonstrate an increase of fluorine in such topically treated teeth (70), the pronounced effect on caries obviously suggests very definite alterations *in vivo* of the properties of enamel surfaces.

Similar to this evidence, is the observation that fluorine in concentrations as low as 1.0 p.p.m. in drinking fluids is effective in reducing the erosion produced *in vivo* on rats' molar teeth by citrate and lactate drinking fluids (71, 72).

Dental caries and fluoride medication.—Experimental fluorination of communal water supplies now in process in numerous communities in the United States and Canada is an attempt to duplicate exposure to natural fluoride drinking waters. Results from these mass population experiments will not be forthcoming for several years, although bacteriological results of somewhat questionable significance have been published concerning one of these studies (73).

The majority of the results following topical applications of sodium fluoride, usually as a 2 per cent aqueous solution, have indicated a significant (upwards of 40 per cent) reduction in dental caries. While the use of fluoride topically thus appears to be a promising caries control measure, variations in the procedure, in the solutions used, etc., need to be investigated (65, 67, 69). Continuity of effect beyond three years (64) is unknown.

McClure (5) estimated the milligram quantities of fluorine being ingested via drinking waters containing 1.00 p.p.m. fluorine to be approximately 1.0 mg. fluorine daily, and suggested the direct addition of 1.0 mg. of fluorine as sodium fluoride daily to children's diets during the first eight years of life as a caries control measure. Although a great number of fluoride tablets have appeared on the market recently for use in caries control, their value has not been demonstrated (74, 75). Most of these tablets contain calcium fluoride, or bone meal, and some provide vitamins, whereas there is no evidence that calcium or vitamins enhance fluorine caries-preventive effects. A fluoride supplement is contraindicated where the drinking water contains at least 0.5 p.p.m. or more fluorine.

The average daily fluorine ingested in food is difficult to evaluate because of the uncertainties, among other things, which surround the fluorine analytical procedure, particularly for organic materials. In general, it would appear that about 0.2 to 0.3 mg. of fluorine is ingested daily in the average adult diet, exclusive of drinking water. It should be emphasized, however, disregarding industrial exposures, that water-borne fluorine is the major fluorine exposure in most human population groups.

There is no indication that edible foods grown in areas where

fluorine in the local water is above normal are increased in fluorine content. Analytical data, fluoride fertilizer studies (76 to 79), and the fact that the majority of fluoride-bearing waters are not surface waters, but come from deep wells, refute this erroneous idea. Thus far it seems unlikely that any of the ordinary edible raw foods are involved in the production of endemic fluorosis, or in the "fluoride-prevention" of dental caries (80). Small increments of fluorine may be added to certain foods when they are cooked in fluoride waters (81). The idea is erroneous that there is an increase of fluorine in cow's milk due to extra fluorine in the cow's ration or drinking water (82). All the evidence available in these regards is negative (83, 84, 85).

Physiological effects of fluorine; effects of fluoride in domestic waters.—Application of results gained from animal experiments on fluoride to effects of fluorine exposures via domestic waters is greatly restricted because of the disproportionate quantities of fluorine involved, variations in species susceptibility and in the duration of the exposure period. Fluorine ingestion from domestic waters in the United States, even in areas of highest water-fluorine concentration (6), would rarely exceed 8 to 10 mg. daily (i.e., less than 0.10 mg. per kg. in the average adult) whereas animal experimental diets usually have provided 5.0 mg. or more fluorine per kg. (86, 87, 88). In a recent study, the growth, hemoglobin, and blood coagulation time were not altered by feeding 5 mg. fluorine per kg., in some instances as long as one year, to 21 litters of dogs involving 99 animals (88). A reduction in blood hemoglobin in rats also receiving 50 p.p.m. fluorine as sodium fluoride in their drinking water was reported by Ginn & Volker (89), but was not confirmed by McClure & Kornberg (90).

The requirement of fluorine in the growing rat seems to be less than 27 μ g. fluorine per kg., which agrees with prior evidence that fluorine probably has no essential function in the rat (91, 92). The metabolism of fluorine in the rat was the subject of an extensive series of studies by Lawrenz, Mitchell & Ruth, and their conclusions, based largely on balance studies, have been summarized by Mitchell & Edman (93).

An attempt to assess the hazard of cumulative toxic fluorosis in human populations was made by McClure & Kinser (94) in an extensive survey of the fluorine concentration of spot urine specimens of high school boys, and of young selectees of the Armed

Forces of the United States. The results indicate that upwards of 90 per cent of natural water-borne fluoride (in concentrations of 0.5 to 4.5 p.p.m. fluorine) is eliminated in the daily urine of teen age boys and young men. Fluorine balance studies (95, 96, 97) furnish additional evidence that the adult human body eliminates the major portion of food and water-borne fluorine when the quantities ingested do not exceed 4.0 to 5.0 mg. fluorine daily. Body sweat may contain appreciable quantities of fluorine which may be influenced by the fluorine being ingested (97). McClure also found negative effects of water containing 0.5 to 4.5 p.p.m. fluorine on the age-height-weight relations and bone fracture histories of high school boys and young inductees (98).

There are a number of reports from fluoride areas outside the United States which suggest toxic effects from excessive quantities of fluoride in drinking waters and perhaps from fluoride contaminated foods. In a discussion of these reports presented elsewhere (85), the opinion was expressed that the fluoride exposure of the afflicted (fluorosed) natives involved in the majority of these studies, exceeds any level of fluoride encountered in the United States; or else other local factors, malnutrition and lack of adequate control groups, discredit the results (99, 100, 101).

Reports of British workers, in particular (99, 100, 102), continue to suggest incipient skeletal effects of fluoride waters, particularly in children from lower economic classes (102). The interpretation of these results is none-the-less difficult because of uncertainties in the fluorine exposure as established either by the mottled enamel diagnosis or by the drinking water surveys. Contrary to Dean and his associates (6), the British workers, for example, regard 1.0 p.p.m. as a high level of fluorine in drinking water, capable of producing moderate to severe mottled enamel. In the United States at least, a content of 3.0 to 4.0 p.p.m. of fluorine in the water supply is associated with an incidence of moderate to severe mottled enamel.

The suggestion that toxic effects of fluorine are influenced by malnutrition may be true, as for example by influencing the quantity of fluoride water ingested (as would probably occur in the absence of milk in the diet). The relation of essential diet factors to human toxic fluorosis must be regarded as a matter of conjecture, although it may be noted from some experimental animal evidence, that dietary calcium above certain minimum

levels impairs the assimilation of dietary fluorine (103). Also, sodium fluoride in excessive quantities has given some evidence of having a variable effect on rats' incisor teeth (104), and on blood calcium and phosphorus, depending on the calcium and phosphorus in the ration (105).

Fluorine and enzyme action.—Borei (106) recently prepared a most comprehensive review concerning fluorine and enzyme activity. His own extensive investigations are confined mainly to the inhibition by fluoride of oxidative processes which proceed by way of the cytochrome system. The capacity of fluoride to inhibit enzymatic processes by forming complex metallic salts is illustrated in a study by Reiner (107). In general, extensive inhibition is obtained by fluorine in cases of enzymes requiring calcium, manganese, magnesium, or iron as catalysts, due to formation of fluorine metal complexes. Fluorine also may combine with prosthetic groups of many phosphoproteins, thus inhibiting activity by formation of fluorophosphoprotein complexes. Enolase is inactivated by formation of magnesium fluorophosphate (108). Sodium fluoride had a strong accelerating effect on the oxidation of cysteine in 0.1 *N* HCl (pH 1.8) but had no effect in less acid solutions (109). The sensitivity of fluorine-enzyme relations to the pH of the medium is emphasized in the report by Fitzgerald & Bernheim (110), where, for example, the oxidation of acetic, heptylic, oleic, and benzoic acids was inhibited by fluorine at pH 6, but not at a lower pH.

Recent years have seen unusual advancements in the chemistry of fluorine and in the appreciation of its scientific and technological value (111). Industrial uses of fluorine have increased accordingly, and with this a corresponding increase in industrial fluorine health hazards. Restrictions of space, however, will not permit discussion of this relation of fluorine to public health.

BORON

Boron is essential for normal growth in higher plants and occurs extensively in animal tissues, but a requirement in animals has not been established. Theresi *et al.* (112) prepared a ration lower in boron than was used previously (113), and although their ration was not adequately consumed, the results suggest that 0.6 μ g. boron per day would probably satisfy the normal growth requirement of the rat.

A relation of potassium to boron metabolism has been investi-

gated (114) as suggested by the evidence that in plant nutrition boron is poorly absorbed from media of low potassium content (115). Due to an extreme potassium deficiency, all the animals in this experiment grew very little (114), but boron supplements seemed to account for longer survival periods, and after 21 days the rats on boron supplements contained 47 per cent more glycogen in their livers than their nonboron controls. Stores of body fat were favored by "liberal supplementation" (114) with boron compounds. The variable food consumption by control *vs.* test groups of rats was not considered. In a similar study, Follis (116) found no differences in heart and kidney lesions of the potassium-deficient rat with or without boron supplements and, contrary to Skinner & McHargue (114), boron had no effect on growth rate or survival time.

Toxic effects of boron were not apparent following an 11- to 16-day boric acid regimen (3 gm. daily) in four normal adults (117). Basal metabolism remained normal (117). In the dairy cow (118), 16 to 20 gm. of borax daily over a 40-day period caused no diuretic effects, loss in weight, or other ill effects. The milk concentration of boron rose from 0.7 p.p.m. to 3.0 p.p.m. which was not regarded as a public health hazard. A retention of boron was not detectable and boron excretion returned rapidly to normal. Oral ingestion and absorption from boric acid, applied in the form of an ointment or a saturated solution, may cause serious and even fatal cumulative boron poisoning (119).

An enzymatic role of boron in plant nutrition was indicated by evidence of an increased rate of oxygen consumption, and a more active polyphenoloxidase, in boron-deficient leaf tissue (120). Borate in 0.01 *M* solution inhibited the activity of tobacco, tomato, and soybean polyphenoloxidase.

COBALT

Numerous experiments continue to suggest the importance of cobalt in cell metabolism, but thus far there is no assurance that cobalt is indispensable to life except in ruminant animals (17, 121). A ration containing as little as 0.0024 p.p.m. cobalt has failed to produce deficiency symptoms in rabbits (122) and cobalt deficiency effects have not been produced in rats (121, 123, 124) receiving rations containing less than 0.03 p.p.m. cobalt daily.

In general, negative results regarding a requirement of cobalt

in nonruminant animals, as well as other evidence, have led to the belief that cobalt does not act directly on the ruminant host but in the rumen itself (125), where microorganisms concerned with the synthesis of some of the B vitamins may be affected (122). In keeping with this idea, Ray and co-workers (126) obtained data which indicate a slightly lower concentration of nicotinic acid, and possibly of the vitamin B₆ group, in the blood of cobalt-deficient sheep.

A recent observation, particularly revealing, is the report by Rickes *et al.* (8), i.e., that emission spectrographic analysis shows the presence of cobalt in vitamin B₁₂. The cobalt-complex nature is regarded as "an outstanding property" (8) of this new vitamin. Since vitamin B₁₂ causes marked hematological responses in pernicious and other anemias, the presence of cobalt in this vitamin may prove particularly enlightening to the question of the role of cobalt in blood chemistry (124, 127, 128).

An exhaustive review of dietary factors (vitamins, amino acids, and minerals, including iron, copper, and cobalt) which are identified with erythropoiesis (128) points out a unique role for cobalt; i.e., a deficiency of cobalt results in anemia, small amounts may produce and larger amounts may depress erythropoiesis. Cobalt polycythemia is readily produced in a number of animal species (129), and a number of reports on this subject have appeared recently (130 to 137). Wintrobe (137) studied the anemia resulting from the inflammation induced by turpentine injection and found it to be largely overcome by cobalt administration. Cobalt polycythemia was developed in rats regardless of the addition of choline or methionine to the diet, but cystine was distinctly inhibitory (136). Although the symptoms of endemic cobalt diseases are not too specific, erythropoietic factors are indicated also by the anemia generally associated with cobalt-deficient ruminant animals.

The role of cobalt in cell metabolism is suggested by its activating effect on arginase (138, 139), as well as by its effective inhibition of growth and respiration in various microorganisms, animal tissues, and tumors. The action of histidine in overcoming some of these inhibitory effects is being studied (140).

Results with radioactive cobalt.—In experimental rats, labeled cobalt appeared in the bile, urine, feces, and liver (141), and intravenously injected radioactive cobalt was detected in plasma, bile, and pancreatic juice (142). Results with radioactive cobalt demon-

strate very poor absorption of the orally administered ion in rats (126). Active cobalt administered intravenously or directly into the rumen is also poorly retained by ruminants (143).

Eighty per cent of orally administered cobalt was eliminated in the feces, and 0.5 per cent in urine (144). The relatively insignificant absorption was indicated by failure to find cobalt in blood, milk, or saliva (144). Radioactive cobalt also was transmitted across the placenta for storage in the liver of the fetus. The bile is an important excretory path for cobalt, 2.0 to 5.0 per cent of injected labeled cobalt being eliminated in 48 to 72 hr., respectively, in bile (145). Analytical data for inactive cobalt (146) likewise suggest that the liver is an important depository for cobalt.

Cobaltous acetate (500 mg. daily) in rations of dairy cows increased the cobalt content of milk from 0.6 mg. up to 2.4 mg. (145). Comar & Davis (146) compared the distribution of injected radioactive cobalt in swine, rabbits, and young calves but found no species differences. Their findings "support the view that the major function of cobalt in the ruminant is a localized action in the rumen, but do not exclude the possibility of a hematopoietic function" (146). In general, the results with radioactive cobalt confirm the belief that the requirement for cobalt by ruminants must be very small indeed, and if required by other animals the quantity is extremely small.

MANGANESE

Manganese-deficiency symptoms and manganese requirement.—

The species relations as regards nutritional requirements and effects of manganese deficiency have been extended by observations on rabbits (147, 148, 149), rats (150 to 154), mice (150), and swine (155). The results indicate growth failure in rabbits, rats, and mice. Congenital debility, testicular atrophy, and sterility, although observed less consistently, appeared in rats and mice deprived of manganese. A decrease in liver-arginase activity was also observed in manganese-deficient rabbits, rats, and mice. While alkaline phosphatase of the bone is less active in the manganese-deficient chick and rabbit, conflicting results are reported for phosphatase activity in the manganese-deficient rat (153, 154). Regarding manganese-deficient rats, mice, rabbits, and swine, in general, the accumulating evidence suggests the occurrence of abnormal skeletal tissues which show certain similarities to the well defined bone deformities in chicks (10). There is the suggestion

that a decrease in alkaline phosphatase activity of bones (156, 157) is a general characteristic of manganese depletion and may explain failures in bone formation. The results of Combs, Norris & Heuser (156) and Smith *et al.* (149) seem to suggest a relation between phosphatase activity of bones and manganese content of the ration. Generally, bone deformities such as "crooked front legs" in rabbits (148) are accompanied by significantly lower values for fresh weight, percentage ash, total ash, and bone density (148, 149). The manganese content of the liver and femur of manganese-deficient rabbits was reduced, but no change in blood serum manganese occurred (148). The value of the above experiments (148) is enhanced by use of the Mitchell paired-feeding technique.

Disagreement is evident in the results of studies on the reproductive ability of manganese-deficient female rats and the viability of their young. Shils & McCollum (10) suggest three distinct reproductive effects of manganese deficiency, i.e., (a) viable but defective young are born; (b) the young die quickly; and (c) a disturbed estrus cycle produces sterility.

Having established the characteristic symptoms of manganese deficiency in the rabbit, Smith *et al.* (149) determined that 0.3 mg. manganese daily was sufficient in the rabbit for purposes of normal bone, phosphatase and arginase activity, but perhaps was insufficient for normal growth. Although lactating dairy cows may retain 150 mg. manganese daily, regardless of the additional quantity ingested, the possibility of a negative manganese balance in dairy cattle is highly remote (158). Manganese in cows' milk is of the order of 20 p.p.m. and the quantity has been increased 2.5 times by feeding manganese sulfate (159).

Physiological effects of manganese.—A suggestion by Rudra (160) that manganese plays a role in the synthesis of ascorbic acid was not confirmed (161). Skinner & McHargue (162), however, presented evidence in support of a role of manganese in the synthesis of hemoglobin in the rat, showing that manganese when combined with iron and copper gave better results than iron and copper alone.

Amdur, Norris & Heuser (163) reported that manganese tended to prevent the deposition of fat in the liver of rats and an interaction between choline and manganese was indicated by the fact that a lipotropic action of manganese was much greater when the choline content of the diet was lowest. Bone fat, as well as liver fat, was reduced by both manganese and choline.

Radiomanganese studies.—Two studies with chicks (164) and rats (165) utilizing radiomanganese confirm evidence of practically complete elimination of manganese through the feces. Both manganese-deficient and control chicks (164) excreted the majority of orally administered manganese. Labeled manganese given orally did not appear in bone, indicating that the manganese requirement of bone may be very small (164). Oral manganese is very poorly absorbed even in the fasting animal (161).

As has proved true in regard to many studies with unlabeled manganese in numerous animal species (166), ingestion of radiomanganese indicates that the liver is particularly active in manganese metabolism (164, 165, 167). From 50 to 75 per cent of the intestinal excretion of labeled manganese was estimated to come from the bile (161). In the chick the role of manganese in liver function could not be identified with arginase (168) since chick liver does not contain this enzyme. When colloidal sols of radiomanganese dioxide were given intravenously to human beings and dogs there was a notable increase in liver manganese (169). In the opinion of these workers (169) the pancreas, as well as the liver, may have special significance in manganese metabolism.

Manganese and enzyme action.—Although increased twentyfold in the diet of rats, manganese had no effect on the activation of cocarboxylase *in vivo*, as measured by the concentration of bisulfite-binding substances in the blood (170). Smith (171) studied the mechanism of manganese activation of L-leucine-aminoexopeptidase and attributed the activation to the formation of a true metal-protein-enzyme compound. Three different methods for the micro-determination of manganese have been reported upon: (a) micro-colorimetric (172); (b) microbiological (173); and (c) a method utilizing the catalytic properties of manganese in an enzymatic oxidation reaction (174).

ZINC

The toxicity of zinc has been demonstrated in many experiments and a comprehensive review emphasizing this subject has been presented (175). Prior to this survey (175) it was surmised that extensive use of zinc in war industries might have produced unsuspected features of zinc toxicity. No new toxic manifestations were noted, however, and the usual precautions in industrial practices utilizing zinc appear to be sufficient.

A requirement for zinc by the rat has been known for some time (176, 177, 178), but this had not been demonstrated in the mouse (179, 180, 181). However, zinc starvation in mice did produce retarded growth, a localized alopecia, and affected liver and kidney catalase (182). Changes in liver esterase, in enamel and dentin, or in the concentration of riboflavin in liver and kidney, did not occur in this animal; nor did any distinctive gross symptoms other than the retardation of growth and alopecia manifest themselves. Food consumption was *ad libitum*, however, and it would be of interest to study these deficiency effects (182) under conditions of controlled feeding.

Numerous experiments have suggested that the pancreas is involved in zinc metabolism (183), but evidence is contradictory regarding the relation of zinc to insulin (185, 186). In support of the view that the acinar portion of the pancreas may be associated with zinc metabolism, Montgomery, Sheline & Chaikoff (187) found 11 per cent of administered radiozinc (Zn^{65}) excreted in the pancreatic juice of the dog in 14 days. Maximum excretion in bile was only 0.4 per cent in eight days. Radiozinc was found in large amounts in the juice obtained from an isolated loop of the duodenum. Sheline and associates (188) injected intravenously minute quantities of zinc in dogs and mice and found by far the largest fraction eliminated via the intestine. In dogs and mice the most active turnover of radiozinc was in the liver, pancreas, and pituitary gland. The least activity occurred in red blood cells, brain, skeletal muscles, and skin (189). The extensive distribution of zinc in the animal body and its presence in intestinal secretions is apparent from these results (188, 189). Vallee & Gibson (190) using an improved dithizone method (191) found normal zinc in blood distributed as follows: red blood cells, 75 per cent; plasma, 22 per cent; and white cells, 3 per cent. These values will serve for comparison of blood zinc levels in blood dyscrasias (190).

Forty-two zinc balances on normal human patients, as reported by McCance & Widdowson (192), led to the conclusion that practically all food-ingested zinc is eliminated in the feces. This agrees with studies in which radiozinc was employed (188, 189). Normally 0.3 mg. zinc per day was excreted in the urine and this quantity did not vary with the intake of zinc either by mouth or by injection. It would appear that the kidney normally has little or no zinc excretory function and does not vary its excretion in proportion to

dietary zinc, or even when the plasma zinc is increased following zinc injections. According to McCance & Widdowson (192), the trace of zinc in the urine is possibly only an end product of a metabolic function of the kidney itself.

Toxicity studies in rats provided Smith & Larson (193) with the evidence that copper and liver tend to alleviate the reduced growth and hemoglobin changes caused by excessive zinc and called further attention to the interrelation and interdependence of mineral elements.

Zinc has shown important relations to numerous enzyme systems. It is present in purified carbonic anhydrase (9, 194, 195) and recent evidence suggests the possibility that purified dehydropeptidase also contains zinc (196). The above results and certain animal experiments (177, 178, 182) suggest that zinc may be an indispensable element, but this has not yet been adequately proven. The possibility of a human need for zinc was remotely suggested in the report by Stevenson (197) that the carbonic anhydrase activity of the blood of premature infants is low. Wide distribution in plants and animals contraindicates a deficiency of zinc occurring in animal or human nutrition.

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CHEMISTRY OF THE HORMONES

BY A. WETTSTEIN AND F. BENZ

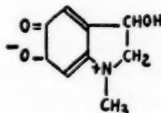
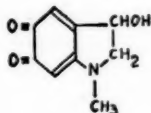
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In the present review reference is made to work carried out in 1948, as well as to a few publications which are intended to characterize the research position at the beginning of the period covered by this report. Only works connected with the chemistry of the hormones, their presence in the organism, and their estimation have been considered. Upon termination of this paper there were, unfortunately, a few journals outstanding, and for this reason, and also in consideration of space limitations, we do not regard this a complete review.

In introduction, we should like to enumerate various extensive monographs published during the past year, which deal in part with the chemistry and physiology of the hormones, namely those of Harris & Thimann (1), Pincus (2), Pincus & Thimann (3), Reifenstein (295), Selye (4), Turner (5), Twombly & Pack (6), Verzár (7), and Zweifach & Shorr (8).

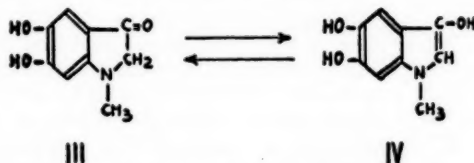
BLOOD PRESSOR PRINCIPLES

Epinephrine.—It was shown in earlier research that, during oxidation of epinephrine into melanin-like substances, a red and unstable pigment adrenochrome (I) is formed, of which the physiological significance is not yet clear. The solubility of adrenochrome



as well as its chemical behavior in reduction led Harley-Mason (9) to postulate for it the structure of a zwitterion (II). During transformation of adrenochrome into melanins, less stable intermediate products are formed and consequently their isolation is very difficult. Employing fluorescence and absorption spectrum

methods, Beauvillain & Sarradin (10) believe they have proved the spontaneous formation of a fluorescent leucocompound (III) in aqueous solutions of adrenochrome. Fluorescence is explained by alternative passage from ketonic into enolic structure (IV).



Whereas oxidation of epinephrine in alkaline solution, according to Barac (11), is not suppressed by thiouracil and its methyl and propyl derivatives, the oxidation of the demethylated epinephrine, arterenol, is inhibited by these goiterogens. 2,3-Dimercapto-propanol is reported by Bacq *et al.* (12) to be a new agent preventing oxidation of epinephrine in aqueous solution, and Konzett (13) found that, by addition of tannic acid, coloring of solutions of epinephrine may be avoided.

Bloch (14) and Pekkarinen (15), as well as Annersten (16), used the fluorescence reaction of epinephrine for its quantitative determination in blood. Brochart (17) demonstrated the occurrence of epinephrine in the sperm of bulls, goats, and men; Chatonnet & Vial (18) estimated the quantity present in the adrenals of rats. Experiments on rabbits by Dodgson *et al.* (19) have shown that *dextro*epinephrine is excreted as a glucuronide. Kaelin (20) synthesized the oxygen analogue of epinephrine. When tested on the narcotized cat, this compound had no action on blood pressure and respiration. The amino analogues of epinephrine and arterenol, both prepared by Duschinsky *et al.* (21), showed only one tenth of the pressor activity of epinephrine.

Arterenol.—Stimulation of sympathetic nerves is accompanied by the liberation of an active principle with sympathomimetic properties. It was first assumed that this neurohormone was identical with epinephrine. Barger & Dale, as long ago as 1910, alluded to the analogy between the effect of sympathetic stimulation of several organs and the action of noradrenaline. Later, several investigators supposed demethylated epinephrine (arterenol, noradrenaline, norepinephrine) to be the adrenergic transmitter. With the aid of biological and chemical methods, Euler (22)

demonstrated the presence of a specific sympathomimetic ergone having the properties of arterenol in various organs and in adrenergic nerve fibers. In further investigations, the same author (23) was able to show that the adrenergic action of extracts of the spleen and of splenic nerves of cattle is predominantly due to the presence of *levo*arterenol, the epinephrine content of such extracts not exceeding 5 per cent. On the other hand, it was shown that the adrenergic action of extracts from certain tissues, such as human coronary arteries and nerves, as well as of extracts from frogs' hearts is produced by epinephrine. According to Bacq (24) the best interpretation of these facts is in the assumption that many tissues synthesize aminated derivatives of catechol and that the synthesis of epinephrine is effected by way of arterenol. Methylation of the nitrogen is, therefore, apparently the last step in the synthesis of epinephrine, as was previously postulated by Blaschko (1942); in certain tissues, however, this methylation does not occur.

Recently Holtz & Schümann (25) found that the hyperglycemic effect of arterenol is approximately one twentieth that of epinephrine. The authors compared the hyperglycemic action of extracts from bovine adrenals with mixtures of epinephrine and arterenol. From these results and from amino nitrogen estimations they conclude that such extracts, in addition to epinephrine, must contain 10 to 15 per cent *levo*arterenol and, furthermore, approximately equal amounts of biologically inert primary amines. According to a paper by Marrazzi & Marrazzi (26) arterenol is not identical with the hypothetical sympathin E. In the light of recent knowledge on the existence of two highly active sympathomimetic hormones, arterenol and epinephrine, the hypothesis by Cannon & Rosenblueth (1937) on the production of sympathin E (excitatory) and sympathin I (inhibitory) within the effector cells can no longer be maintained. With the intention of retaining the term sympathin to denote any active sympathomimetic ergone liberated from "adrenergic" axones, Euler (27) proposes the terms sympathin N and sympathin A for the transmitters of arterenol (noradrenaline) and epinephrine (adrenaline) respectively.

A paper by James (28) deals with the description of a simple method for determining arterenol, epinephrine, and methyladrenaline, singly or in mixtures, by combining an enzymatic and a chromatographic method. It is supposed by Tainter *et al.* (29)

that the naturally occurring arterenol has the *levo* configuration since L-isomers are generally more active. The authors succeeded in obtaining optically pure preparations of *levo*- and *dextro*arterenol by resolution of the racemate of arterenol with D-tartaric acid. *Dextro*arterenol had indeed only 3 to 4 per cent of the pressor activity of the *levo* form, a ratio similar to that of the epinephrine isomers.

Other pressor principles.—Raab *et al.* (30) extracted a sympathomimetic amine from human and animal brain tissue. It was not identical with epinephrine or arterenol and was named encephalin. By means of ethanol fractionation, Olsen *et al.* (31) obtained from the arterial blood of hypertensive patients, but not from that of normal subjects, preparations with pressor action in the rat assay. Rapport *et al.* (32, 33) succeeded in isolating a crystalline sulfate of a base, serotonin, from cattle serum, the vasoconstrictor activity of which was twice that of commercial epinephrine. Plasma obtained from cats and dogs after intravenous injection with semirefined extracts of cat and other animal kidneys caused, according to Shipley & Helmer (34), sustained pressor responses in nephrectomized cats, dogs, and rats. Hase (35) prepared from the anterior lobe of ox pituitary a dialyzable pressor principle, which was not identical with vasopressin and epinephrine but was capable of normalizing the blood pressure of adrenalectomized rats. Earlier investigations by Shorr *et al.* (36) have established the presence of a vasodepressor substance (VDM) of hepatic origin in the blood of animals during the hyporeactive phase of shock, which substance has been found to participate in the regulation of the peripheral circulation. Mazur & Shorr (37) have now succeeded in concentrating VDM from extracts of animal and human liver and have identified it by immunochemical procedures as ferritin.

THYROXINE

In order to clarify the relationship between chemical structure and physiological activity, Frieden & Winzler (38, 39) prepared a number of compounds, structurally related to thyroxine, by alteration of the side chain and also by modification of the prime ring, and tested them both for thyroactivity and for thyroxine antagonism. In accordance with the conclusions drawn by the authors, the ortho dihalogenophenolic diphenyl ether configuration, a

hydroxy group ortho or para to the ether oxygen, and a side chain which includes a functional group are structural requirements for thyroxine-like activity in amphibia and mammals. The glycine homologue of thyroxine, 3,5-diiodo-4-(3',5'-diiodo-4'-hydroxyphenoxy)-DL-phenylglycine, synthesized by the same authors (40), proved to be one third as active in the amphibian metamorphosis test and only 1/500 as active in preventing the increase of the thyroid weights of thiouracil-fed mice. Leblond & Grad (41) estimated the thyroxine-like activity of chloro and bromo thyronine derivatives. The results emphasize the prominent rôle of iodine for thyroxine-like activity; their complete or partial replacement by bromine or their partial substitution by chlorine reduced, but did not eliminate, biological activity. According to Harington (42), the sulfur-containing analogue of thyroxine, 3,5-diiodo-4-(4'-hydroxy)-phenylsulfido-phenylalanine, when assayed on tadpoles of *Xenopus laevis*, showed approximately one fifth of the metamorphosis-accelerating activity of thyroxine. The physiological activity of the optically active isomers of thyroxine was studied clinically by Rivers & Lerman (43). D-Thyroxine was found to have an activity between one eighth and one tenth of that of the L-antipode. Saul & Trikojus (44) investigated the behavior of DL-3,5-diiodo-4-hydroxyphenyllactic acid under conditions similar to those which were found to result in the conversion of diiodotyrosine to thyroxine, and isolated a crystalline compound which they believe to be the lactic acid analogue of thyroxine.

Winikoff & Trikojus (45) found N-diethyl-sulfanilamide to be a reagent for the colorimetric estimation of thyroxine. Methods for labeling thyroxine with radioactive iodine were reported by Frieden *et al.* (46). Hutcheon (47) presented two methods for biological assay of thyroid activity. One is based upon the decreased resistance of thyroxine treated mice to anoxia, the other upon an acute weight loss occurring in rats during the two days following the administration of thyroxine. A bioassay of thyroid activity, based upon the eruption of the fore limbs in tadpoles of *Xenopus laevis*, has already been described. Hamilton *et al.* (48) modified this method by using the appearance of the fore limb buds as a criterion.

Roche *et al.* (49, 50, 51) made quantitative investigations on the formation of thyroxine and its precursors, as well as on the diminution of tyrosine, due to the action of iodine on casein,

zein, and thyroglobulin. A low concentration of iodine merely caused the formation of monoiodotyrosine which, when the iodine content was increased, was transformed into diiodotyrosine; afterwards this compound was oxidized to thyroxine. By means of further oxidation processes, thyroxine was destroyed again. Casein was more easily and more strongly iodinated than thyroglobulin but in both proteins the quantity of thyroxine formed was closely related to the tyrosine content. Thyroxine formation via chemical iodination of proteins does not only depend on the content of tyrosine but is also a function of the position of this amino acid within the peptide chain of the protein molecule. Roche & Michel (52) tried to obtain further information on the mechanism of thyroxine formation by investigating the iodination of di- and tripeptides containing tyrosine. Blocking of the carboxyl and the amino group of tyrosine did not influence thyroxine formation. It would appear, however, that the nature of the substituting group, i.e. the length of the carbon chain, is of decisive importance, since iodination of L-leucyl-L-tyrosine gave a higher yield of thyroxine than glycyl-L-tyrosine. Earlier investigations by von Mutzenbecher and others have shown that aerobic incubation of diiodotyrosine under mildly alkaline conditions results in the formation of thyroxine. In strongly alkaline solutions, Pitt-Rivers (53) demonstrated, in addition to thyroxine, the formation of 3,5-diiodo-4-hydroxy-benzaldehyde. The iodine content of pure thyroglobulin may be variable and is not characteristic. Roche *et al.* (54, 55) therefore assumed that, in thyroglobulin formation, a protein of uniform composition is first formed by the thyroid, and then transformed into iodinated products.

It was formerly supposed that the minute iodine content of plasma is due to the presence of a small quantity of thyroglobulin. Results obtained by Taurog & Chaikoff (56) favor thyroxine rather than thyroglobulin as the probable constituent of blood plasma. Fink & Fink (57), with the aid of radioiodine, investigated the metabolism of iodine by the thyroid and demonstrated the presence of monoiodotyrosine in thyroid hydrolysates of rat and man. Frieden & Winzler (58), using the goiter-prevention method, compared the parenteral thyroxine-like activity of natural and synthetic thyroproteins. Natural thyroid proteins consistently showed more thyroxine-like activity than could result from their L-thyroxine content, whereas synthetic products revealed only one half the activity expected from their L-thyroxine content.

PROTEIN HORMONES

Insulin.—Lens (59) prepared absolutely pure insulin with an activity of 26.8 ± 0.8 I.U. per mg. The molecular weight of insulin calculated from osmotic measurements was found by Gutfreund (60) to be $48,000 \pm 3,000$. A paper by Robinson (61) deals with the absorption of water vapor on insulin and plasma albumin. According to earlier investigations of Waugh, by heating at pH 2, insulin may be converted into highly asymmetric fibrils. Waugh (62) and Waugh, Smith & Fearing (63), using sulfuric acid, phenol, and other agents, succeeded in regenerating from such fibrils crystalline products resembling native insulin. Nitrobenzene and chlorobenzene, as well as some salts, inhibited regeneration with phenol. It must be concluded from these findings that only small structural changes take place during fibril formation and the process is, therefore, one in which globular or corpuscular units are linked endwise. The stability of the fibrils is of considerable importance and places insulin in a rather unique position in protein chemistry. Du Vigneaud *et al.* (64, 65, 66), in studying the mechanism of action of mustard-type vesicants with proteins, treated insulin with benzyl- β -chloroethylsulfide and *n*-butyl- β -chloroethylsulfide, containing radiosulfur, and isolated a crystalline insulin-benzyl- β -chloroethylsulfide which, when tested on rabbits, displayed considerable hypoglycemic activity. Evidence was obtained that a fraction of the vesicant is attached to the free amino group of some of the phenylalanine residues in the intact protein molecule.

Volkin (67) observed that thiocyanate increases the solubility of insulin in its isoelectric region. This fact suggests an interaction of the reagent with certain basic groups of the protein. The specific activity of insulin has been related on previous occasions to the occurrence of guanidino residues (arginine) in the hormone molecule. It is well known that quantitative determination of the guanidino groups of proteins by means of the Sakaguchi reaction covers only part of the total arginine present. It has now been found by Mourgue (68) that, with regard to this reaction, insulin does not differ from other proteins.

An insulin inactivator, capable of inactivating the hormone during *in vitro* incubation and having the properties of an enzyme, was extracted from animal and human livers by Broh-Kahn & Mirsky (69). Using extracts from the livers of normal and alloxan-treated rats, these authors (70) also showed that insulin

exerts no direct effect on the enzyme systems known to be concerned with the glucose-glycogen cycle of the liver. Mirsky *et al.* (71) elaborated a method for detecting minute amounts of insulin in human urine and estimated the daily excretion of the hormone by normal subjects to be 0.16 ± 0.04 units.

One of the most urgent problems of protein chemistry is the elucidation of the sequence in which amino acids are arranged in protein molecules. In previous work, Sanger, by use of the reagent 1,2,4-fluorodinitrobenzene, had shown that the submolecule (mol. wt. 12,000) of insulin is made up of four peptide chains which appear to be held together by disulfide links provided by cystine. By oxidation of these disulfide links with performic acid the insulin could be split into its separate polypeptide chains. Sanger (72) has now isolated from oxidized insulin two fractions which he further investigated by the use of the fluorodinitrobenzene reagent. On the basis of the cysteic acid content of the fractions and on the assumption that the insulin submolecule is built up of four parallel peptide chains, the author discussed the arrangement of the chains in the insulin molecule and showed that only two alternatives are possible.

Woolley, in an earlier paper, found in the partial hydrolysates of various proteins, and especially of insulin, a growth-promoting factor for *Lactobacillus casei* which he called "strepogenin." He studied (73, 74) tryptic digestion of the dinitrophenyl (DNP)-insulin and fractionated pancreatin digests of DNP-insulin and of the DNP derivative of oxidized insulin. The strepogenin structure appears to occur at the end of a peptide chain in the insulin molecule, some of the free amino groups of insulin belonging to the growth factor. By fractionation of the pancreatic digests of insulin, a series of peptides was isolated. Some of these peptides were also obtained from digests of DNP-trypsinogen; it may therefore be concluded that there is identity between a sizable portion of the two proteins.

Butler *et al.* (75, 76) also investigated the action of enzymes on insulin. The authors observed that pepsin transforms insulin into fragments with a molecular weight of approximately 2,000. The action of chymotrypsin on insulin occurs in two stages. In the first stage, a number of smaller peptides are removed from the end of the insulin chains, leaving a homogeneous protein-like residue, a "core," with a molecular weight of approximately 5,000. This res-

idue contained the greater part of cystine and probably all the amino acids existing in insulin, and, in the second stage of digestion, was slowly hydrolyzed by the long continued action of chymotrypsin.

Glycogenolytic (hyperglycemic) factor.—Clinical observations as well as investigations on animals have given rise to the suggestion that pancreatic tissue may produce, besides insulin, another factor which, on intravenous injection, causes initial hyperglycemia. It is assumed that this glycogenolytic factor is elaborated by the α -cells of the islets of Langerhans. Using cysteine-inactivated preparations of insulin, Zimmermann & Donovan (77) found that the factor influences the blood sugar level only through its action on the hepatic glycogen reserves; it does not appear to neutralize the hypoglycemic action of the insulin. Commercial insulin preparations, as well as crystalline insulin, may contain this principle, Heard *et al.* (78) obtaining products which caused liver glycogenolysis and hyperglycemia from the supernatant solution of the first isoelectric precipitation of insulin. A study of the distribution and origin of the glycogenolytic factor was made by Sutherland & de Duve (79, 80). Of all tissues investigated, only pancreas and parts of the gastric and intestinal mucosa contained it. Extracts of fetal calf pancreas especially were found to possess maximal glycogenolytic activity, whereas extracts from pancreas of rabbits made diabetic with alloxan showed the same activity as those from normal animals but caused hyperglycemia without subsequent hypoglycemia. The authors, as well as Sutherland & Cori (81), therefore suggested that the factor is a protein.

Adrenocorticotropic hormone.—Giroud & Martinet (82, 83) implanted in rats cells of the basophilic zone of beef and hog pituitary and observed hypertrophy of the adrenals, as well as stimulation of follicle growth in the ovary. This confirms earlier results on the formation of adrenocorticotropic and follicle-stimulating hormone in the basophilic tissue of the anterior pituitary. Wells (84) obtained experimental evidence which shows that adrenocorticotropic hormone is already produced by the hypophysis of the fetal rat. By analogy this may serve to explain the relatively enormous size of the suprarenals of the human fetus.

The administration of adrenocorticotropic hormone to rats is followed by a temporary decrease in the amount of ascorbic acid present in the adrenals. Under suitable experimental conditions,

the degree of diminution is a function of the dose of hormone administered. This relationship was adapted by Sayers & Sayers (85, 86) to a quantitative assay of adrenocorticotrophic activity. Cooke *et al.* (87), employing hypophysectomized rats, used this test for assay purposes in body fluids such as urine and plasma.

Wilhelmi & Sayers (88) reported corrections of data published earlier on the electrophoretic mobility of adrenocorticotrophic and parathyroid hormones. The new values are in substantial agreement with those given by Li *et al.* (89).

Growth hormone of the pituitary.—The term "chondrotrophin" to designate the growth hormone of the anterior pituitary is preferred by Dingemans *et al.* (90) instead of Selye's "somatotrophin" (4). Kemp (91) has discussed the possibility of using dwarf mice for the estimation of growth hormone. Gjeddebæk (92) likewise examined the accuracy of various methods for determining the potency of growth hormone preparations and recommended the growth-plateau technique in the female hypophysectomized rat method because of its relatively high accuracy and simplicity.

By fractionation with ethanol at low temperatures, Wilhelmi *et al.* (93) isolated a crystalline and electrophoretically homogeneous preparation of bovine anterior pituitary growth hormone. This product also exhibited the highest glycostatic activity of all glycostatic extracts obtained up to now. It appears, therefore, that glycostatic activity is one of the properties of the growth hormone. Later on, Li *et al.* (94), with a technique similar to that of Wilhelmi *et al.*, succeeded in crystallizing a pure preparation of growth hormone prepared some time ago. By the aid of ultracentrifugal experiments, Li & Moskowitz (95) measured the molecular weight of a homogeneous extract of the growth hormone from ox pituitaries. The value, 44,000, is in fair agreement with that obtained previously by other methods. According to a communication by Li *et al.* (96) on iodination of growth hormone in aqueous solution, only four out of eleven tyrosine residues were transformed into diiodotyrosine; more iodine was bound by iodination in urea solution. It was found that the activity of iodinated growth hormone preparations decreased as the uptake of iodine increased, and the authors therefore concluded that tyrosine in the hormone molecule is essential for its growth-promoting activity.

Gonadotropic hormones.—Experiments with cell fractionation of sheep anterior pituitary gland showed that gonadotropic activity

is heavily concentrated in the large granule fractions, whereas nuclei possess only a low activity, probably caused by adherent large particles [Catchpole (97)]. According to Sims & Bishop (98), gonadotropic potency of anterior pituitary of *Rana pipiens* undergoes seasonal variation; between January and March the activity increases. Bischoff (99) investigated the effects of urea denaturation on unfractionated sheep pituitary gonadotropin; assays were made of the gonadotropic, antagonism, and antihormone properties. The author concludes from his experiments that the multiple physiologic properties manifested are inherent in a single substance, but that the specific effects are dependent upon the spatial arrangement of specific adsorption foci (secondary valence effects) rather than upon the molecular structure in the usual sense.

Chorionic gonadotropins.—By means of cytochemical investigations, using a staining technique for mucopolysaccharides and glycoproteins, Pearse (100) was able to demonstrate the production of the gonadotropic hormones of the basophilic cells of the pituitary as well as of the Langhans' cells of the chorionic villi and of chorionepithelioma. Stewart *et al.* (101) observed chorionic gonadotropin secretion from human placenta grown in continuous tissue culture *in vitro*. Pedersen-Bjergaard *et al.* (102, 103) determined the gonadotropic substances of the urine of normal and pregnant women. Up to puberty, gonadotropic activity is only rarely detectable. The authors confirm the increase in output of gonadotropin during pregnancy, especially the enormous augmentation of urinary gonadotropin in the second month of pregnancy. An equally high but brief rise in the output appears towards the end of gestation, a fact overlooked by earlier investigators. Hamburger (104) found that the concentration of chorionic gonadotropin of the afternoon urine is equal to that of the morning sample.

The hyperemia reaction of the rat ovary has been employed for several years for rapid determination of pregnancy. Papers by Riley (105) and by Zondek & Sulman (106) deal with experiences on the practical application of this test. According to Kupperman *et al.* (107) the hyperemia reaction is produced not by the follicle-stimulating hormone, but only by luteinizing or luteotropic hormones, and therefore may be used for a rapid estimation of the latter factors in gonadotropic preparations.

Nalbandov & Baum (108) employed stilbestrol-inhibited cocks for qualitative and quantitative assay of gonadotropic prepara-

tions. Determination of changes in comb size and testis weight, as well as histological examination of the testes, makes it possible to distinguish between follicle-stimulating and luteinizing hormone and mixtures of both.

According to studies of Dorfman & Rubin (109), the assay of chorionic gonadotropin from human pregnancy urine and serum may well be made possible by the use of the uterus weight curve of immature rats, since a rectilinear relationship between the logarithm of the dose and the response of the uterus exists. Based on the stimulation of spermatogenesis in rats, following the administration of gonadotropic preparations, Milco & Pitis (110) elaborated a method for the standardization of chorionic gonadotropin. Pregnant mare's serum was tested by Dorfman *et al.* (111) by means of the response of the chick's testis.

In recent years the significance of the pregnancy assay with the South African clawed toad, *Xenopus laevis* Daud, the so-called Hogben test, has been accentuated by the need for a rapid and reliable method for human pregnancy assays. The oviposition, induced a few hours after administration of chorionic gonadotropin in mature toads, is the criterion for a positive result. Galli Mainini (112) detected emission of spermatozoa in urine of the South American toad *Bufo arenarum* Hensel after injection of pregnancy urine and, for the first time, recommended the use of the male anura for the pregnancy assays. These findings were confirmed by several authors (113 to 122) who, in part, also used the males of other amphibian species for a rapid diagnosis of pregnancy.

Claesson *et al.* (123) claim to have succeeded in obtaining a crystalline and electrophoretically homogeneous preparation of chorionic gonadotropin, assaying 6,000 to 8,000 I.U. per mg. In hypophysectomized rats, this preparation caused only stimulation of the interstitial gland of the ovary but showed no influence on follicle growth and maturation, even when large doses were administered. In women, sufficiently high doses stimulated development of Graafian follicles, but no luteinizing effect could be observed.

STEROID HORMONES

An interpretation of steroid nomenclature, useful to the uninitiated, was given by Mason (124). Fieser & Fieser (125) proposed possibly final changes and their nomenclature is therefore em-

ployed in this paper.¹ Jaffe (126) has suggested use of the prefix *seco* to denote the scission of polycyclic systems (e.g., 16,17-*seco*-estrane).

In the elucidation of the constitution of steroids, comparison of their molecular rotations is often very advantageous. Barton and co-workers (127, 128) compiled tables of rotation contributions for the functional groups and double bonds in different positions and discussed the anomalies observed [cf. Djerassi (129)]. Ultraviolet and infrared spectroscopy, widely used for the characterization of steroid hormones, was reviewed by Jones *et al.* (130), who contributed many new data to the infrared part.

Of general biological assay methods, both the nuptial coloring and the ovipositor lengthening test, using *Rhodeus amarus* Bloch, are now found to be totally unsuitable (131).

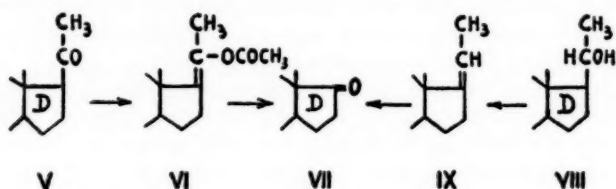
Attempts to achieve the total synthesis of steroid hormones or sterols without any benzene ring have apparently been intensified, the crucial point lying in the great number of possible stereoisomers. Thus Bachmann *et al.* (132) and Cook *et al.* (133) prepared three-ring structures, containing functional groups for the attachment of the fourth ring but hitherto having no substituent in the ring A. Such a substituent, however, is contained in the still partially aromatic end product of Grob and co-workers (134). Cornforth & Robinson (135), who synthesized a tricyclic degradation product of cholesterol, have probably approached this final objective most closely.

Androgens.—The biochemistry of androgens, including assay and isolation, has been exhaustively reviewed by Dorfman (see 3). Dorfman himself (136) worked on the bioassay method, employing inunction of the chick comb. An investigation of the color reactions of androgens with antimony trichloride [cf. Pincus (280)], bismuth and aluminum trichloride was published by Clark & Thompson (137). Dirscherl's colorimetric method, using sulfuric

¹ The letters α and β are used without parentheses to designate the configuration of a substituent at a nuclear center of asymmetry. Arbitrary trivial indexes are used with quotation marks [e.g., " α "-estradiol = estradiol-17 β]. The letters α and β are also used for established configurations at C₂₀ (which makes an additional convention necessary), arbitrary configurations in the side chain being designated as a and b (e.g. pregnandiol-3 α , 20a). Differences in configuration from that of a typical steroid are ordinarily characterized by the prefix *iso*, whereas the prefix *allo* is reserved for use in connection with C₄ and the prefix *epi* for cases where the orientation of a hydroxyl is concerned (e.g. dehydroepiandrosterone).

acid, was adapted by Nielsen to the quantitative determination of dehydroepiandrosterone and testosterone (138). Miescher & Kägi (139) elucidated the chemical structure of the primary products in their color reaction for 17α -hydroxy-steroids, the pseudoandrostenes. Hilmer & Hess (140) reported the ultraviolet spectrophotometric estimation of androsterone and testosterone 2,4-dinitrophenylhydrazones after separation from the hydrazones of estrone and progesterone by chromatography.

Gallagher *et al.* (141) described the degradation of several 20-keto-pregnanes (V) to the corresponding 17-ketones (VII) by preparation of the 20-enol-acetates (VI) followed by ozonolysis.



Sarett (142) introduced, *inter alia*, 17: 20 double bonds as well (IX), starting from 20-hydroxy-pregnanes (VIII) and subjecting a further number of them to the tosylate tertiary amine method, followed by oxidation to the 17-ketones (VII). One of the four possible isomers at C_{16,17} of 16-methyltestosterone was prepared by Julian and co-workers (143) from dehydroepiandrosterone through the 16-methylene derivative. Its androgenic activity, after intramuscular injection, proved to be one eighth to one tenth that of testosterone. Hershberg *et al.* (144) described the synthesis of 16-C¹³-dehydroepiandrosterone acetate while MacPhillamy & Scholz (145) prepared 17-(methyl-C¹⁴)-testosterone. Such hormones containing tagged atoms should prove extremely useful in metabolism studies. A cycloheptadecane-1,9-dione, analogous to androstenedione, but possessing neither ring connections nor methyl groups, showed neither androgenic nor estrogenic activity [Ruzicka *et al.* (146)].

For the relation of activity to constitution in the androgens and other sex hormones, reference may be made to the review by Miescher (147). According to Colonge *et al.* (148), even purified progesterone would still have a very slight androgenic activity. Dingemans and co-workers (149) returned to the "X-substances"

in the acid fraction of testis or urine, which enhance the effect of testosterone.

From the incubation suspension of Δ^4 -androstene-3,17-dione with surviving rabbit liver slices, Clark *et al.* (150) have now obtained 11.5 per cent of testosterone and traces of epitestosterone and unidentified steroids. Sweat & Samuels (151) find that cozymase and citrate are important factors in the enzymatic destruction of testosterone by minced liver tissue; the first enhances the primary breakdown to 17-ketosteroids, the second affects a yet unidentified metabolic pathway.

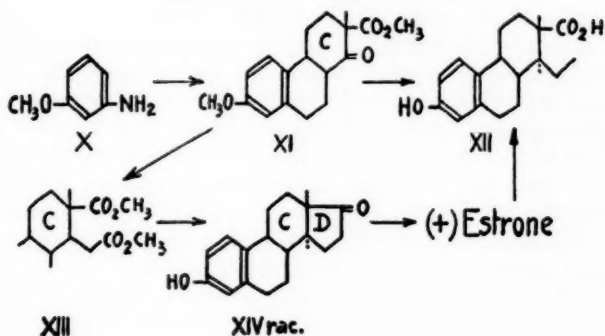
Estrogens.—The biological, physical, and chemical methods for the assay of ovarian hormones were reviewed by Pincus (3), the chemical methods also by Marrian (152). Marrian suggested that the nonestrogenic urinary chromogens in the Kober reaction could be measured separately by prolonged heating, while Jayle *et al.* (153) determined all the phenolsteroids together by means of a modified Kober method. Contrary to former suggestions, 16-ketone estrone cannot be part of the chromogen in the classical Kober procedure [Marlow (154)]. The new color reaction of Masquelier & Jaubert (155) makes use of the stable methylglyoxal reagent without heating. Jailer (156) has described a fluorometric method for estrone plus estradiol, suitable for human but less so for stallion urine, and similar work has been done by Finkelstein (157). Friedgood *et al.* (158) published comprehensive papers on the identification and quantitative microdetermination of estrogens by ultraviolet absorption spectrophotometry, including the separation of the androgens and of estriol from the estrone-estradiol fraction by distribution between two solvent systems. Quantitative analysis of estrogen mixtures was also accomplished by Carol and co-workers (159), who investigated the benzenesulfonyl esters in the infrared. Dorfman *et al.* (160) gave a new bioassay method, not very sensitive but of high precision, based on the weight of the chick oviduct. The comparative activities in this test were: stilbestrol 100, estradiol benzoate 48, methylbisdehydro-doisylnolic acid 42, estradiol 18, and estrone 12.

Pearlman (293) reviewed the chemistry and metabolism of the estrogens in general, whereas Miescher (147, 161) summarized the work of his group on doisylnolic acids and related compounds. As regards the various sources of estrogens, Bartlett *et al.* (162) and Curnow *et al.* (163) extracted phenolic estrogenic fractions from

young rapidly growing grass and from Australian subterranean clover. Reversing the procedure of some well-known experiments, Brand and co-workers (164) exposed testicles of guinea pigs to a supernormal temperature but did not get an increased estrone production. Werthessen *et al.* (165) demonstrated the presence of a labile alcoholic ketonic estrogen in human and rabbit blood.

The partial synthesis of 16-ketoestrone and 16-keto-" α "-estradiol has now been described in detail by Huffman & Lott (166). Velluz & Muller (167) prepared the 17-methylketone of the estrone type. Djerassi (168) found that 17-ketosteroids, like estrone and androsterone, are readily sulfonated in the 16-position. Nevertheless, Djerassi & Scholz (169) could transform 1,4-androstadiene-3, 17-dione into 1-methylestrone, then into 1-methylestradiol and, by fusion with alkali, into the, also inactive, 5-methyldoisynolic acid with an opened ring D. Inhoffen's beautiful estradiol synthesis from cholesterol proved to be impracticable in the coprostan series (170). The products obtained by synthesis and from urine are absolutely identical (171). Heer & Miescher's stepwise chemical transformation of equilenin into bisdehydro-doisynolic acid through the corresponding bisdehydro-marrianolic acid, involving opening of ring D, could then be performed under much milder conditions (172). Here again, the natural hormone yielded not the highly active but the estrogenically inactive (+) β acid, apparently evolved without rearrangement and therefore most likely possessing the same trans configuration as equilenin. The direct proof of the configuration, by conversion of the bisdehydro-marrianolic acid into a 1,4-dicarboxylic acid, was not possible according to Billeter & Miescher (173). From the literature, however, Shoppee (174) compiled some other chemical, and Klyne (175) some physical evidence (molecular rotation differences), showing that (+) equilenin and (+)estrone almost certainly possess the same *trans* fusion of rings C/D. Hausmann & Smith (176) prepared globulin complexes of estrogenic acids with a view to determining their immunological properties.

The most outstanding success in the chemistry of steroid hormones was achieved with the total synthesis of natural estrone by Anner & Miescher (177). The approach of several groups to this problem, difficult to solve because of the 16 possible stereoisomers, and the realization of the synthesis were reviewed by Miescher (178). The decisive step proved to be the isolation of three of the



four tricyclic ketoesters (XI) of the octahydrophenanthrene series in crystalline form from the oily mixture, prepared according to Bachmann (281) from *m*-anisidine (X) [cf. also Robinson (282)]. One of these sterically homogeneous racemates, the ketoester A, had been converted previously by Anner & Miescher (283) into the "natural" (+)doisynolic acid (XII), obtainable from estrone. Following classical lines, they have now succeeded in adding the five-membered ring D to ketoester A (XI), through the two racemic methyl-marrianolic esters (XIII), which yielded two estrone racemates. The one with *cis* configuration of the rings C/D was estrogenically inactive, but the *trans* compound (XIV), highly active. The latter has been resolved and the (+)estrone, obtained after a total of about 28 steps, proved to be identical in every respect with natural estrone. The conversion of estrone into " α "-estradiol and estriol is already known. In a 20 step total synthesis, a racemate of a structural and stereochemical isomer of estrone, with the keto group at C₁₆ instead of C₁₇, was prepared by Wilds & Johnson (179). Its estrogenic activity proved to be low, whereas a racemic 8-dehydro derivative, a 16-keto isomer of isoequilin A, was somewhat more active.

Contrary to the situation in the four-ring estrogens, only those mono- and bisdehydro-doisynolic acids which contain the ethyl and carboxyl group in the "unnatural" *cis*-position are estrogenically active. Heer & Miescher (180) have now prepared such monodehydro-marrianolic and -doisynolic acids by diene synthesis, via corresponding hexahydrophenanthrene-1,2-*cis*-dicarboxylic acids, whereas Breitner (284) had obtained structural isomers of them.

The monodehydro-marrianolic acid was transformed, in analogy with the previous examples, into the 8-monodehydro-isoestrone (*cis*), which, as expected, showed a very low activity. Hogg (181) obtained from Hagemann's ester lower homologues of the 8-monodehydro-doisylnolic acid, with a methyl instead of an ethyl side chain. It is most likely that the configurations he attributed to his products have to be reversed. Approaches, similar to Miescher's, towards the four-ring *cis*-estrogens, were investigated independently by Bachmann *et al.* (182), who prepared a structural stereochemical isomer of estrone from one of the diene adducts of the monodehydro type. In the bisdehydro series, only structurally "wrong" adducts originated. A new total synthesis of bisdehydro-doisylnolic acid was accomplished by Johnson & Graber (183), utilizing a Stobbe condensation. Weidlich (184) described experiments which should have led towards a new synthesis of equilenin [cf. Bachmann (285)].

Whereas the doisylnolic acids contain three of the four original rings of the natural estrogens intact, in the allenolic acids a further ring (C) is open. Horeau & Jacques (185) compared several types of compounds and their activities, the most active substance of the allenolic series being the α,α -dimethyl- β -ethyl-allenolic acid, which, however, is still considerably less active than bisdehydro-doisylnolic acid. Desoxyallenolic acids have approximately one tenth of the activity of the methoxylated compounds. Wieland & Miescher (186) also observed a loss of activity when changing from an allenolic acid to the corresponding aldehyde and carbinol or to the tetrahydro-allenolic acid (with hydrogenated ring B). Finally Horeau (187) prepared a hydroxyphenyl-propyl-cyclopentanone with a remote analogy to estrone but open rings B and C.

In defining the constitutive field of estrogenic activity, Miescher (147) stated that all of the more active estrogens have two oxygen-containing groups situated at opposite ends of an aromatic or partially aromatic system of 2 to 4 rings, which are not even always condensed. Segaloff (188) arranged some substances, tested by subcutaneous injection in rats, in the following order of decreasing estrogenic potency: methyl bisdehydro-doisylnolic acid, sodium bisdehydro-doisylnolate, " α "-estradiol [17 β], Westfeld's lactone acetate, " β "-estradiol [17 α], and estrolo-lactone acetate. Courier *et al.* (189) adduced proof that the order of activity may change according to the target tissue examined. Peder-

sen-Bjergaard & Tønnesen (190) compared estradiol monobenzoate with the dipropionate in rats and mice, whereas Trentin *et al.* (191) found that much of the former may be destroyed by ultraviolet irradiation. In addition to the usual estrogenic effects, Wolff & Wolff (192) observed with bisdehydro-doisyonic acid a transformation of the right gonad into an ovary in both sexes of chicken embryos.

Of estrone acetate injected into bile fistula dogs, 14 to 35 per cent could be biologically accounted for by Pearlman and co-workers (193) in the bile, considerably more than in the urine and faeces, but only a small quantity was isolated as estrone and " α "-estradiol. Accordingly, Twombly *et al.* (194) found, after injection of equilin brominated with radioactive Br⁸², much radioactivity in the gall bladder and the bile, but no selective localization in the many tissues tested. The inactivation of estradiol by rat liver is obviously enzymatic and oxidative (195), the heat-stable component of the enzyme being cozymase (196). This inactivation is prevented by exhaustion after a restricted diet (197). The inhibition *in vitro* of crystalline kidney alkaline phosphatase by estradiol or estrone is said to be very slight, but very strong by some of their phosphorylated derivatives (198). Estrone slows down considerably the oxidation of subcutaneous fats or triolein *in vitro* (199). A summary of his studies on vitamin B-estrogen interrelationships was given by Hertz (200).

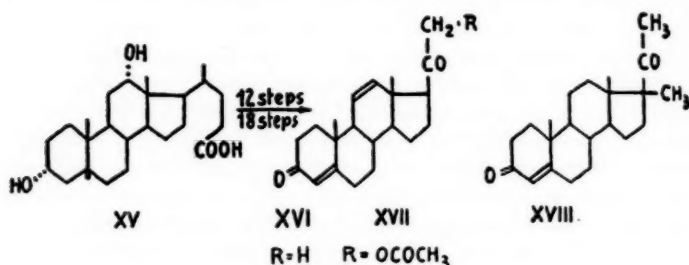
The many papers on estrogens of the stilbestrol type unfortunately cannot be dealt with here as space is limited.

Gestagens.²—A gravimetric procedure for the estimation of progesterone, based on the reaction with excess 2,4-dinitrophenylhydrazine, has been described by Klein and co-workers (201). Djerassi (202) showed that a normal bis-hydrazone, which can be split by pyruvic acid and hydrogen bromide, is produced. Their very sensitive intrauterine injection method enabled Hooker & Forbes (203) to determine, in a pregnant woman and in animals, 4 to 8 μ g. progesterone per ml. of whole blood, located entirely in the plasma. For the first time progesterone was found, with this method, in the plasma of a bird (hen) as well.

The chemistry and metabolism of progesterone has been reviewed very thoroughly by Pearlman (286), the synthesis of

² For nomenclature compare (147).

steroids of the progesterone series by Ehrenstein (204). The latter author also revised the naming of a number of compounds described previously. Radioactive 21-C¹⁴-progesterone was prepared by two groups, Riegel & Prout (205) and MacPhillamy & Scholz (145). Plattner *et al.* (206) synthesized 14,17-diisoprogesterone, which, according to their hypothesis, is a higher homologue of Ehrenstein's active 10-norprogesterone, but it proved entirely inactive. A surprise in this field was the result published by Meystre, Tschopp & Wettstein (207). Using the simple degradation method for bile acid side chains to methylketones with N-bromosuccini-



mide, they transformed desoxycholic acid (XV) into 11-dehydropregesterone (XVI) [cf. Reichstein (287)]. With a subcutaneous threshold value of 0.2 mg. in the rabbit uterus test, this substance proved to be three times as active as progesterone and is therefore the most active gestagen known. To the nine compounds possessing considerable progestative activity, Plattner and co-workers (208) have recently added a new one: 17-methyl-pregesterone (XVIII) which, subcutaneously in rabbits, is about twice as active as progesterone.

Progesterone, when applied locally to the skin, was shown to be as effective as by subcutaneous injection (209). The liver proved to be an important site for the inactivation of progesterone: Pearlman & Cerceo (210) isolated 0.5 to 2 mg. per liter each of pregnanol-3 α -one-20, pregnanediol-3 α -20b and etiocholanediol-3 α ,17 β from unhydrolyzed gall bladder bile of cows in advanced pregnancy.

Adrenal cortical hormones.—The comparatively complicated chemistry and metabolism of adrenal cortical hormones (ACH) has been reviewed by Heard (288). Staudinger & Schmeisser (211)

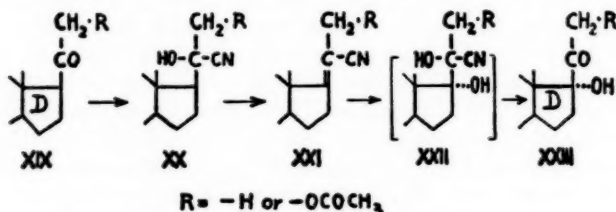
described a method for the chemical assay of ACH, especially from adrenals, based apparently on the method of Heard & Sobel (289). After concentration of the hormones by solvent partition, the difference of the reduction values with phosphomolybdic acid is estimated, before and after the destruction of the hormones by alkali. Corcoran & Page (212) and Daughaday *et al.* (213) determined the corticosteroids (from urine and plasma), after oxidation with periodic acid, by colorimetric measurement of the formaldehyde liberated and distilled from the reagent mixture. Furchgott and co-workers (214) presented the infrared spectra which contain specific, strong absorption bands.

Fieser & Fieser (125) dealt with the configuration of corticosteroids asymmetric at C₂₀. All the known natural compounds, with the exception of Reichstein's substance O, contain a 20 β -hydroxyl. Reichstein *et al.* (215) published several further examples of the degradation of digitaloid lactones to corticoid ketols, often obtaining good yields. Lardon (216) recently transformed periplogenin into desoxycorticosterone and progesterone by this method. Bergmann & Stevens (217) worked towards the conversion of ergosterol to ACH by side chain degradation and introduction of a double bond in ring C. The high effectiveness of 11-dehydroprogesterone (XVI) caused Meystre & Wettstein (218) to prepare the analogous Δ^{11} -anhydrocorticosterone acetate (XVII), employing their new procedure for the direct degradation of bile acids to the ketol side chain.³ In two different ways, they obtained from desoxycholic acid (XV) a new substance which was not identical with that described by Shoppee (290), showing, on the contrary, the expected properties, the chief of these being an effectiveness in survival tests at least as high as that of desoxycorticosterone. Simultaneously von Euw & Reichstein (219) described another partial synthesis of the compound (XVII), starting from the corresponding etio acid. The constitution of the product was assured by degradation and by conversion into 11-dehydrocorticosterone acetate, this representing the latest and sixth approach to this hormone. An isomeric desoxycorticosterone, with the double bond in position 1 instead of position 4 (rings A/B *trans*) and one fifth of the activity of the natural substance, was obtained by Djerassi *et al.* (220).

³ Compare last year's review [*Ann. Rev. Biochem.*, **17**, 369 (1948)].

Wilds & Shunk (221) found an important method for the preparation of acid chlorides from α,β -unsaturated keto acid sodium salts with oxalyl chloride and applied it to Reichstein's classical diazoketone synthesis of desoxycorticosterone. In a new process for the conversion of methylketones into ketols, Ruschig (222) condensed pregnenolone with oxalic ester, proceeded to the 21-iodo derivative and from there to the 21-acetoxy derivative. The mechanism and the stereochemical result of the Serini reaction (conversion of a 17,20-diol-20-acetate with zinc into a 20-ketone) was discussed by Shoppee (223). Mattox & Kendall (224) applied a modified method for the introduction of a 4,5-double bond in 3-ketosteroids to the preparation of 11-dehydrocorticosterone acetate and its 17 α -hydroxylated derivative. Bisnor-steroid acids were degraded by Julian and co-workers (225) via the Curtius reaction to 20-amino derivatives, and then by the Hofmann reaction to Δ^{20} -pregnenes. These are interesting intermediates for corticoid ketols, yielding etio acids by ozonization and 20,21-diols by hydroxylation. Besides double bonds in position 20, MacPhillamy & Scholz (226) introduced, also by the Hofmann reaction, double bonds in positions 11 and 22 of steroids.

Sarett (227) discovered an elegant method for the hydroxylation in position 17 of 20-ketopregnanes (XIX), by which their



cyanhydrines (XX) are dehydrated (XXI) and then hydroxylated (XXII), hydrogen cyanide being spontaneously split off to yield the 17 α -hydroxy-20-ketopregnanes (XXIII). As this procedure is also feasible for 20-keto-21-acetoxypregnanes, it permitted a new synthesis of Kendall's compound E. Another, the third method of preparing 17 α -hydroxypregnanes, corresponding in their configuration at C₁₇ with the natural ACH, was published by Plattner *et al.* (228). These authors found that the 16 α ,17 α -oxide of $\Delta^{16,3\beta}$ -acetoxy-20-keto-allopregnene, which cannot be hydro-

generated catalytically, is reduced by lithium aluminum hydride to produce Reichstein's compounds J and O.

Pregnenolone, implanted in adrenalectomized guinea pigs, was unable to keep the animals alive (229). Induced by exercise and thermal stimulation, an excretion of 40 to 80 μ g. of reducing adrenal corticoids in sweat, has been observed (230). Surviving adrenal cortical tissue produced cholesterol *in vitro* from sodium acetate containing C¹⁴ (231). The glycogen formation from glucose in the surviving diaphragm of the rat, which is increased by insulin, was inhibited by desoxycorticosterone (232).

Urinary steroids.—As it is not always possible to locate the origin of these steroids, they are dealt with separately. The interest in this subject is still very strong, as may be seen from the number of publications. Lieberman & Dobriner (233) summarized their beautiful chemical and physical work on steroid excretion, postulating the existence of a third type of conjugates besides the sulfates and glucuronidates. A comprehensive symposium on the assay of urinary steroids took place (234).

Neutral 17-ketosteroids of urine.—The chemical estimation of 17-ketosteroids was reviewed by Callow (234), the biological assay of urinary androgens and estrogens by Emmens (234), and the clinical significance of such assays by Bishop (234). Mason (235) summarized his extensive studies on urinary steroids in adrenal disease and the metabolism of adrenal hormones, while Dorfman (236) dealt with the metabolism of androgens and Engstrom (237) with the nature and significance of the neutral steroids in urine.

Hamburger & Rasch (238) elaborated the optimal conditions for hydrolysis and extraction in a micromodification (1/50 of a 24 hr. specimen) of the Zimmermann-Callow method. Butt and co-workers (239) described an improved micromethod for the quantitative separation of 17-ketosteroid extracts into 3 α and 3 β -hydroxy fractions, which were estimated polarographically. The last fraction is known to be important in the diagnosis of adrenal cortical carcinoma. Polarographic and chemical methods (e.g. Zimmermann reaction) were compared by Morris (234). A new colorimetric assay for dehydroepiandrosterone (DA), using furfural plus sulfuric acid and indicating the presence of real or potential unsaturation in ring B combined with a hydroxyl group or double bond in ring A, was published by Munson *et al.* (240). Lieberman *et al.* (241) studied the acid sulfates and sulfites of several steroids

and their cleavage, proceeding without Walden inversion. Shoppee (242) adduced further direct proof of the β -orientation of the hydroxyl group in cholesterol (therefore also in DA), and of the chlorine atom in the urinary chloro-ketone from DA.

According to DeKoning and co-workers (243), the excretion of total 17-ketosteroids in normal female rabbits increased from 1.5 mg. per 48 hr. at an age of 5 months to 4 mg. at approximately 2 years, and was not changed after oral administration of sodium estrone sulfate. Kimeldorf (244) ascertained in the neutral ketonic fraction of male rabbit urine 2.2 mg. of 17-ketosteroids per 48 hr., which decreased by 41 per cent after castration; implantation of testosterone pellets then caused a fourfold increase. Only about 2 per cent of the neutral ketonic fraction was androgenically active [Davis *et al.* (245)]. Hamilton & Hamilton (246) found that, in normal men, the daily excretion diminished with increasing age from 25 to 72 years from 10 mg. α - and 1 mg. β - to 2.8 mg. α - and 0.1 mg. β -hydroxy-ketosteroids. Two different groups (247, 248) determined the reduction in the excretion of 17-ketosteroids in man during starvation. According to Furuhielm (249), excretion in healthy women was generally between 4 and 8 mg. per 24 hr., occasionally higher in cases of virilism and especially of Cushing's syndrome, lower in cases of metropathia haemorrhagica, where the estrogenic substances were increased. Reitman (250) found lower 17-ketosteroid values after bilateral prefrontal leukotomy. Klotz *et al.* (251) with spasmophylic men, and Jayle & Bret (252) with hypertensive women. The excretion in adrenal cortical tumor cases was investigated by Kepler and co-workers (253), who isolated 9 steroids, by Dingemanse *et al.* (294), by Leahy & Butsch (254), and by Nielsen *et al.* (255), in the latter studies special attention being paid to the β -hydroxy fraction, mostly DA.

Dobriner *et al.* (256) have now described in detail their methods for the quantitative estimation and the isolation of neutral steroids from human urine. With this procedure 42 ketosteroids have been isolated, namely 35 α - and 7 β -ketones, 26 of which could be completely identified, including 14 that had not been isolated previously from human urine; of these, 4 were new steroids. All compounds were saturated or unsaturated androstane, etiocholane, pregnane, 17-isopregnane, or allopregnane derivatives. For their identification and for the control of the fractionation, methods of infrared spectrometry have been developed, the differences be-

tween the spectra being most significant at the lower frequencies [see also (130)]. Barton & Klyne (257) identified a new 17-ketone, found by Dingemanse *et al.* (258) in the urine of a girl with adenoma of the adrenal cortex, as *i*-androstene-6-ol-17-one, this being the first *i*-steroid from a natural source.

Dorfman *et al.* (259), after the injection of androsterone, isolated 2 per cent of epiandrosterone from urine, whereas after injection of the latter, 8 per cent of androsterone and 6.7 per cent of Δ^2 - or Δ^3 -androstenone-17 were found. The application of DA gave rise to minute amounts of etiocholane-3 α ,17 α -diol; in Mason's (235) experiments to etiocholane-3 α -one-17 and androsterone. The same excretion products were isolated by Devis & Férin (260) after implantation of testosterone. Through incubation of these two 17-ketosteroids as hemisuccinates with surviving liver slices, Schneider & Mason (261) obtained as chief products the corresponding diols and diones, whereas the incubation of DA yielded, in addition to the Δ^5 -androstene-3 β ,17 α -diol a small amount of the 3 β ,16 β ,17 α -triol. The partial synthesis of the last substance has been described by Huffman & Lott (262). Henriques *et al.* (263) and Hoffman *et al.* (264) have investigated the excretion of ketosteroids, androgens, and pregnanediol, following the administration of various hormones, also when given orally. The synthesis of the urinary Δ^9 -etiocholen-3 α -ol-17-one was accomplished by Sarett (265). Grauer *et al.* (266) presented evidence of potentiation and depression of varying proportions of androsterone and DA in the chick comb-weight test.

Estrogens of urine.—The enhanced estrogenic activity of urine extracts prepared by zinc-hydrochloric acid hydrolysis, whereby estrone and hypothetical estrogen metabolites are reduced [Smith & Smith (291)], has been confirmed by Stimmel (267) and by Bishop (234). For this treatment, a special quality of zinc dust is indicated [Smith (268)]. According to Rosenmund (269), the result of biological assays after hydrolysis is dependent on the presence of reducing agents preventing inactivation. Addition of 0.1 per cent of ascorbic acid before hydrolysis has therefore been recommended. The assay of extracts from urine of pregnant women was discussed. A simple method for the extraction of estrogens from carbonized urine residues has been described (270). After injection of 16-keto-estrone in a man, estriol was found in his urine by Stimmel *et al.* (271).

Pregnane derivatives of urine.—The chemical assay of urinary pregnanediol was reviewed by Haslewood (234). Sommerville *et al.* (272) described a procedure, based on that of Astwood (292), for a reasonably accurate estimation of more than 0.4 mg. of pregnanediol in a fifth of a 24 hr. sample of urine, and modified this technique so that the method became suitable for routine determination within 3 hr. of amounts greater than 5 mg. per 24 hr. A rapid method was also published by Rabinovitch (273), using zinc dust-acid hydrolysis with adsorption of the pregnanediol to the zinc. An improved general method for the extraction of steroid and other sulfates from mares' urine has been elaborated by Klyne *et al.* (274), through which sulfates of Δ^{18} -allopregnene- 3β -ol-20-one, of allopregnane- 3β -ol-20-one and of a uranediol were obtained.

Decourt *et al.* (275) identified pregnanediol glucuronide in the urine of gynecomastic men. A glucuronide of pregnane- 3α , 17-diol-20-one was isolated by Mason *et al.* (276) from the urine of a female pseudohermaphrodite, and a large amount of 17-hydroxycorticosterone from the urine of a case of Cushing's syndrome with severe diabetes mellitus. According to de Watteville *et al.* (277), the excretion of pregnanediol by women in postmenopause, after injection of progesterone, is increased 50 per cent by saturation of the organism with vitamin E. Dorfman and co-workers (278) were able to demonstrate the absorption of progesterone by men, after oral administration, by recovering from the urine 15 per cent of the substance in the form of pregnanediol glucuronide and 2 per cent of pregnanolone glucuronide. No such isolation was possible after high oral doses of anhydro-hydroxy-progesterone. After administration of large amounts of 11-dehydrocorticosterone to patients with Addison's disease, Mason (279) found pregnane- 3α , 20-diol-11-one in the urine, the 17-ketosteroid excretion remaining the same.

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FAT SOLUBLE VITAMINS^{1,2}

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VITAMIN A

CHEMISTRY

Achievements in the field of synthetic vitamin A chemistry are reviewed by Milas (1) and Heilbron (2). Synthetic vitamin A acetate, is now marketed by one company in this country (3), and two other companies are also in production (4).

Karrer & Benz (5) have prepared the hydrocarbon analogous to vitamin A and named it axerophthene. This compound is biologically active although its lower homologues are entirely without vitamin A potency, and Karrer concludes that the entire C_{20} atoms arranged in a specific manner are essential for vitamin A activity but that the substituents on the terminal carbon atom are of little significance. Meunier (6, 7) objects to the use of axerophthene for the vitamin A hydrocarbon and suggests dihydroaxerophthene. The term axerophthene should be reserved to designate anhydrovitamin A. Furthermore, he reports that the vitamin A hydrocarbon prepared by him is biologically inactive. Karrer and co-workers (8) also claim that β -carotene is a by-product in a dehydration reaction which converts vitamin A into anhydrovitamin A. However, Meunier (6) contends that the compound is probably the ether of two molecules of vitamin A rather than β -carotene.

Kitol obtained from mammalian liver oil and also vitamin A-active compounds obtained from kitol by heat processing have been patented (9, 10). Apparently kitol, $C_{40}H_{58}(OH)_2$ with an absorption maximum at $286\text{ m}\mu$ and no biopotency, consists of two molecules of vitamin A combined in such a fashion that upon heating at low pressures one molecule of vitamin A is formed from each molecule of kitol. Alpha particle bombardment with radon also effects the conversion of kitol to vitamin A (11).

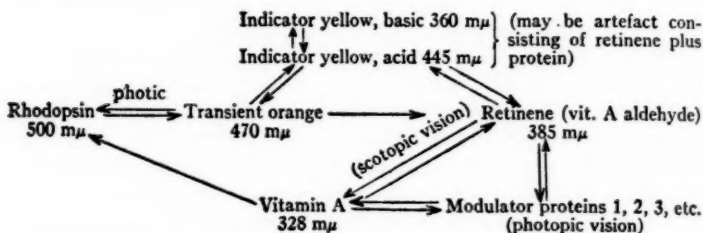
The preparation of vitamin A from β -carotene *in vitro* with a 30 to 40 per cent theoretical yield by oxidation with osmium tetroxide has been reported by Goss & McFarlane (12). Hunter

¹ This review covers the period from December, 1947 to December, 1948.

² Communication No. 149.

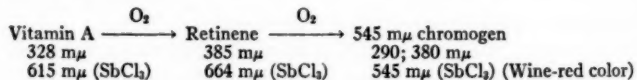
and co-workers (13) have made x-ray diffraction studies on β -carotene and its oxidation products through the stage of β -carotenone and conclude that the usually considered formulae for β -carotene and its oxidation products are correct. A new book on carotenoids by Karrer & Jucker (14) is worthy of mention because of its authoritative and comprehensive coverage.

Visual processes.—Three papers, one by Bliss (15) and the others by Morton and co-workers (16, 17), describe the chemistry of the visual process, and a composite concept of the process can be outlined as follows:



Morton and associates (18) have devised a simple method of preparing vitamin A aldehyde from vitamin A in excellent yield. The compound has been crystallized and fully characterized. Meunier (19) carried out a mild oxidation of vitamin A alcohol with potassium permanganate and obtained a considerable yield of vitamin A aldehyde, or axerophthal in his terminology, and also some 5, 6-epoxide of axerophthal.

Wald (20, 21) has also confirmed the formation of vitamin A aldehyde from mild oxidation of vitamin A, using a clever procedure in which the reaction is carried out on a chromatograph column of manganese dioxide. In the presence of excess vitamin A retinene is released from the adsorbent surface as soon as formed. When vitamin A is limited, retinene is further oxidized to a new substance as follows:



The 545 $m\mu$ chromogen is a polyhydroxy-carbonyl compound, two molecules of which, Wald believes, may condense to form an important prosthetic group of a photosensitive visual pigment.

Compounds related to vitamin A.—Retinene₂, the corresponding aldehyde of vitamin A₂, which enters into the visual cycle (porphyropsin→retinene₂→vitamin A₂) of fresh-water fish retinas, may be prepared from vitamin A₂ by mild oxidation. Also, both vitamin A₁ and retinene, when subjected to an Oppenauer reaction, give a C₂₀ aldehyde which appears identical with retinene₂. Morton *et al.* (22) infer from this that vitamin A₂ differs from vitamin A by having one additional conjugated double bond situated in the ring. This agrees with the structure attributed to the C₂₀ aldehyde by Heilbron (23). Shantz (24), however, prepared crystalline vitamin A₂ and presents evidence that it possesses a structure analogous to the lycopene structure in carotenoids as postulated by Karrer (25). The biological activity of the crystalline vitamin A₂ prepared by Shantz is 40 per cent that of crystalline vitamin A₁. Vitamin A₂ (26) was stored in the liver in the form of vitamin A₂ esters with no evidence of conversion to vitamin A₁.

The biological fate of anhydrovitamin A was also studied (27). It was found deposited in the liver not as anhydrovitamin A but as a new compound possessing a hydroxyl group. This "rehydrovitamin A" was extracted and fed to vitamin A-deficient rats and possessed 10 to 20 times the activity of the original anhydrovitamin A. Apparently, the rat was able to rehydrate one of the double bonds of anhydrovitamin A but was incapable of rearranging the remaining double bonds to the normal vitamin A position.

Vitamin A methyl ether has been synthesized (28, 29) and proved to have the same growth potency as vitamin A₁ for vitamin A-deficient rats. It has also been reported to have vitamin A-like activity in preventing hyperplasia and hyperemia of the thyroid gland caused by thiouracil (30).

PHYSIOLOGY

Factors influencing the *in vivo* conversion of β -carotene and its biological activity relative to vitamin A have properly been the object of considerable study this past year. Koehn (31) reported that β -carotene is quantitatively converted to vitamin A by the rat if adequate quantities of α -tocopherol are ingested. This supports the theory that conversion of β -carotene to vitamin A occurs by fission of the molecule yielding two molecules of vitamin A and not by a random oxidation reaction that inactivates half of each carotene molecule. Koehn's results are difficult to reconcile with

most existing data concerning the relative potencies of carotene and vitamin A. For example, the potency attributed to the new U.S.P. Reference Standard is predicated on the assumption, based on numerous bioassays, that 0.3 $\mu\text{g.}$ of vitamin A equals one unit, either U.S.P. or International (32). By definition, 0.6 $\mu\text{g.}$ of β -carotene equals 1 I.U. Perhaps the feeding levels of both carotene and vitamin A in Koehn's experiments were high enough to induce a near-maximal growth response, making it difficult to distinguish between real differences in potency.

McCoord & Clausen (33) measured the very rapid appearance of vitamin A, *per se*, in blood and other tissues of vitamin A-depleted rats following administration of carotene, and thus have confirmed the previously announced finding that a major site of conversion of carotene to vitamin A in the rat is in the intestinal wall (34).

Wiese and co-workers (35, 36) studied the conversion of carotene to vitamin A in rats with hypofunctioning thyroids due to thiouracil feeding. Although the extent of maximum growth after vitamin A or carotene feeding was markedly depressed by hypothyroidism, the dose required to induce a 50 per cent response was unaltered, being 0.5 $\mu\text{g.}$ of carotene and 0.3 $\mu\text{g.}$ (1 I.U.) of vitamin A. However, it is also contended (37, 38) that carotene conversion *in vivo* is directly proportional to intensity of thyroid function, from practically nil in hypothyroidism to supernormal in hyperthyroidism. The altered carotene metabolism in thyroid dysfunction is not due to changes in the basal metabolic rate *per se* but is brought about by some other physiological action of the thyroid gland (39). The thymus gland is apparently not implicated in vitamin A metabolism (40, 41). Thymectomy on as many as five generations had no influence on the sensitivity of response of rats to deficiencies of vitamin A, D, or protein.

Although liver storage tests (32, 42) are finding increasing favor as a bioassay procedure for vitamin A, they should be used with caution since there are many factors which may influence the utilization and storage of vitamin A by the animal. For example, lutein interferes with the deposition of vitamin A following the administration of either vitamin A or carotene (43). This effect is not due to decreased absorption. Furthermore, compared with β -carotene, cryptoxanthin is twice as potent in causing storage of vitamin A as in promoting growth. Conversely, α -carotene is

twice as potent for growth as for liver storage (44). Johnson & Baumann (45) also showed that tocopherol fed at relatively high levels simultaneously with carotene reduces the quantity of vitamin A stored.

In line with current views on the necessity of good maternal diets for health in the offspring, Warkany & Roth (46) found that rats on diets deficient in vitamin A before and during pregnancy produced offspring which have a fibrous membrane behind the lens of the eye, retrolenticular fibrosis, and other ocular abnormalities. The lungs, heart, and most of the soft tissues examined were underdeveloped and abnormal. Supplementation of the diet with carotene decreased the incidence of malformed young and, coincidentally, increased fertility.

Evidence was presented by Mayer & Krehl (47) that a decrease in the gross efficiency of food utilization, which seems to be a reliable index of vitamin A deficiency, appears sooner than any other symptom. They (48) have also demonstrated that increased amounts of fat in the diet seem to be protective against vitamin A deficiency as shown by an increased survival time. Vitamin A-deficient rats on synthetic diets showed signs of acute scurvy, which were correlated with reduced blood and liver ascorbic acid levels, and enlarged, vitamin C-low adrenals; the scurvy could be relieved by 50 mg. doses of vitamin C (49, 50). Conversely, Loosli and co-workers (51) found in foxes and mink that extra dietary vitamin C increased vitamin A storage and plasma levels, and concluded that vitamin C biosynthesis depends on the animals' resources of vitamin A. However, Mapson & Walker (52) found that decreased food intake in vitamin A deficiency in the rat was sufficient to reduce vitamin C levels in body tissues. Furthermore, vitamin C synthesis could be stimulated by chloretone to the same extent in the vitamin A-deficient as in the normal rat. Thus, they conclude that there is no relationship between vitamin A status and capacity to synthesize ascorbic acid. Guerrant (53) added vitamin C to the standard vitamin A-deficient bioassay diet and found no change in growth response.

The physiological significance of cholesterol remains an intriguing mystery which Williamson (54) links with vitamin A. Fetuses from cholesterol-fed rats contained significantly less vitamin A than those from controls, presumably because of failure of placental transport of vitamin A from the maternal circulation to the fetus.

Lipid metabolism is also affected in the frog by vitamin A (55), including an accumulation of cholesterol in liver and kidneys in the vitamin A-deficient animal. This recalls the report by Villaverde & Vidal (56) that vitamin A regulates the level of cholesterol in human blood.

Brown & Morgan (57) report that vitamin A is essential for the growth of tissue protein but not for its maintenance. Efficiency of protein utilization for growth was decreased in young vitamin A-deficient rats while nitrogen metabolism was unaffected in adult animals.

Interrelationships between the metabolism of vitamin A and the function of various hormones are still controversial. New evidence supports the view that vitamin A is antagonistic to the thyrotropic hormone (58, 59). Truscott (60) reports a direct action of vitamin A on the growth and development of the ovary which would undoubtedly be reflected in its function. However, estrogen injections failed to affect significantly the plasma or liver vitamin A in rabbits (61). In insects there is evidence (62) that carotenoid metabolism is related to reproductive function. Astaxanthin, the principal pigment of the young locust, is entirely replaced by β -carotene at the time of sexual maturity. As previously discussed, thyroid function may (37, 39) or may not (35, 36) be involved in carotene conversion. At least, the plasma level of vitamin A and carotene seems to be dependent on the level of thyroid function (63, 64).

Vitamin A in the nutrition of domesticated animals.—Tom (65) has emphasized the need for vitamin A supplementation of dairy cows under conditions of so-called practical feeding. The health of the cow and also of the calf is markedly improved when additional vitamin A is given during the later stages of pregnancy and during the first 30 days after calving. This need for extra vitamin A is described by Alvarez (66) in South America where dairy cows may become so deficient that corneal ulceration is noted, normal gestation interrupted, and abnormal calves are produced. Injection or feeding of vitamin A supplements quickly cures such abnormalities. Parrish *et al.* (67) found the alcohol and esterified forms of vitamin A, given orally during the terminal stages of gestation, equally effective in fortifying the vitamin A reserves of the neonatal calf. Also, both forms of vitamin A are utilized more efficiently than β -carotene (68) as measured by increase in vitamin A in the blood plasma.

Madsen and co-workers (69) reported an interesting study on bulls. Fertility could be established in those animals receiving 50 μ g. or more of carotene per kg. of body weight daily, while bulls maintained on rations providing less vitamin A showed rapid decrease in sexual activity and ability and a marked increase in the percentage of abnormal spermatozoa with progressive decline in motility. Injections of ascorbic acid failed to benefit the condition but carotene feeding restored the bulls to apparently normal activity and semen production. Histological evidence of testicular injury due to vitamin A deficiency was evident even 20 months after resumption of carotene feeding. Salisbury (70) found contrary evidence in six dairy bulls on diets low in carotene for 16 months. No clinical manifestations of vitamin A deficiency or noticeable impairment of semen production were induced. Further experiment, in which the roughage component of the ration was changed from hay to dried beet pulp, resulted in development of incoordination, edema, capillary hemorrhage, and a gradual increase in per cent of abnormal spermatozoa with concurrent decrease in motility. These symptoms were reversed only in part when carotene supplements were given in oil. Further degeneration of the germinal epithelium was prevented but there was no consistent improvement in semen characteristics. Sterility in horses which could be overcome by parenteral administration of vitamin A has also been reported (71).

Foxes and mink require approximately 25 I.U. per kg. of body weight per day which can be supplied by adding 10 I.U. of vitamin A per pound of moist diet (49). Keane and co-workers (72) found that the normal plasma levels of vitamin A in dogs are between 300 and 600 I.U. per 100 ml. The amount of carotene present is slight and does not interfere with the color reaction for vitamin A. Also, Rouir (73) has found that vitamin A is evenly distributed throughout the different lobes of the liver in dogs.

Pierce continued his studies on the vitamin A metabolism of Australian sheep (74). Newborn lambs had very low reserves of vitamin A but ingestion of colostrum rapidly increased the blood and liver levels to normal. External signs of vitamin A deficiency in the rabbit were manifested (75) only when the animals received large doses (10 to 20 mg. per day) of tocopherol. Only then was xerophthalmia induced and a decrease of vitamin A in the liver and blood effected. The testicular degeneration observed was not related to the ocular lesions. Histological examination revealed

that destruction of the spermatogenic elements and other lesions of the reproductive system resembled those of vitamin E deficiency

In the bobwhite quail, Nestler and co-workers (76) have found that carotene is utilized only one-third to one-seventh as efficiently. as vitamin A alcohol, one-half to one-tenth as effectively as natural vitamin A ester, and only one-fourth to one-seventeenth as effectively as vitamin A acetate, based on vitamin A storage in the liver. The optimum amount of carotene for young quail was about 900 $\mu\text{g.}$ per pound of feed.

Hansborough (77) developed a clever technique of administering vitamins by injection into hens' eggs after 24 hr. incubation and studied the influence of various quantities of vitamin A and D and of liver extract upon the chick embryo. Vitamin imbalances produced had toxic effects upon the embryo, such as delay in its development and abnormalities of the nervous and vascular systems. Johnson and co-workers (78) found 100 $\mu\text{g.}$ of vitamin A per 100 gm. of feed satisfactory for growth in chicks from 2 to 16 weeks of age.

Grummer (79) has studied the vitamin A and vitamin C plasma levels in newborn pigs and found that the former was low, approximately 10 $\mu\text{g.}$ per 100 ml., while the vitamin C level was relatively high. Mature pregnant sows showed the reverse.

Absorption.—Freedman (80) and Süllmann (81) have succeeded in preparing stable aqueous dispersions of vitamin A and of carotene which are suitable for oral or parenteral administration. Tweens (such as polyoxethylene sorbitan monolaurate) were used as the solubilizer. Most efforts in the past to prepare water-soluble forms of fat-soluble vitamins have failed because the surface-active agents used as emulsifiers have also lysed the red blood cells in the body. A more fundamental approach would be to develop derivatives of vitamin A which dissolve in water in true solution, as suggested by Milas (82).

Serum vitamin A in normal children rose to higher levels more rapidly following a test dose of an aqueous emulsion of vitamin A than when fish liver oil concentrate was used (83). Patients with cystic fibrosis of the pancreas acquired serum vitamin A levels, following a test dose of water-soluble vitamin A, which approached those of normal children who were given fish liver oil concentrate. Other confirmatory reports (84 to 93) seemingly establish the fact that vitamin A in aqueous preparations is absorbed by humans significantly better than when dissolved in oily media.

Esh and co-workers (94, 95) have shown that the same improvement in utilization of vitamin A and carotene occurs in the rat and in the cow particularly when soybean lecithin was fed. Scharf (96) confirms these results with soybean lecithin. Adlersberg and co-workers (97) report that lecithin promoted intestinal absorption of vitamin A in sprue but not in cases of severe liver damage. Halpern *et al.* (98) tried methocel as a solubilizer and found that vitamin A oils, whether fresh or oxidized, had greater biological value for chicks when fed in water emulsion than in the original oil solution. This phenomenon is not specific for vitamin A or for carotene, as Jones *et al.* (99) found an increased absorption of triglycerides due to the influence of emulsifying agents in the diet. Absorption of dietary cholesterol in the rabbit is also favorably influenced (100) when given with a surface-active agent such as Tween.

Of the many factors which influence absorption of vitamin A, those associated with aging are most important. Rafsky & Newman (101) studied 43 individuals between the ages of 69 and 89 years old. Vitamin A absorption tests on these aged subjects showed relatively limited and slow absorption through the intestine. In the majority of cases, blood vitamin A and carotene levels were below normal. Mahle & Patton (102) observed that mineral oil and a hydrophilic mucilloid laxative reduced the absorption and availability of minimal amounts of vitamin A and carotene.

Storage.—Quantitative aspects of the storage of vitamin A were investigated by Davies & Moore (42). Single doses of 60,000 units of vitamin A resulted in storage of about 80 per cent of the dose in the liver. Higher intakes caused undesirable responses and less efficient storage. The upper limit of concentration of vitamin A in rat liver after high doses of oral vitamin A seems to be around 20,000 I.U. per gm. and thus of the same order as that found in the liver of polar bear, seal, whale, and certain sharks. Glover & Morton (103) have re-examined vitamin A storage in the pyloric caeca of halibut. The surprisingly high concentration of vitamin A is explained on the basis that during the absorption of the fatty acids and vitamin A from the lumen of the pyloric caecum, a lipase continually effects alternate hydrolysis and synthesis of all lipid esters as they pass through the mucosa and tunica propria layers. Both vitamin A esters and glycerol esters tend to accumulate because of their slow rate of removal in the relatively underdeveloped lymph system of the halibut.

Stability.—Saliva and gastric juice of babies from birth to four years of age, when incubated with vitamin A, destroyed up to 65 per cent of the vitamin, with the greatest amount of destruction occurring within the first five minutes (104). Boiling the saliva and gastric juice did not affect this property.

The stability of vitamin A in aqueous Tween preparations was superior to that of the same vitamin A diluted in cottonseed oil (93), even in the presence of 2 per cent tocopherol and 4 per cent lecithin, a good antioxidant combination for oil. Ethanolamine plus ascorbic acid is recommended by Russian workers (105) for the preservation of vitamin A in oil.

A number of papers and patents during the year have been concerned with the preparation of vitamin A concentrates from fish liver oils by various means (106 to 114). Ultraviolet irradiation of milk caused no vitamin A destruction (115). Reports that there is no significant difference between the vitamin A content of raw and of pasteurized milk (116) have been contradicted (117) by the finding that pasteurized milk had lost 20 per cent of its carotene and 19 per cent of its vitamin A.

CAROTENE

A patent by Wall & Kelley (118) describes the production of pure carotene by chromatographic procedures which eliminate the contaminating chlorophyll and xanthophyll. Carotene destruction in harvested vegetable material is an important economic problem. The mechanism consists of two phases, an enzymatic and a photo-destructive phase, which proceed independently (119). Methods for reducing the loss of carotene in plant materials have been studied by several workers (120, 121). Relatively high moisture content, organic acids, and replacement of oxygen by carbon dioxide in the container are all important factors.

Ezell & Wilcox (122) have studied the storage of carotene in sweet potatoes and find that there is an absolute increase in the content of this pigment during curing and storage after harvesting. Other workers (123) have found that plant materials which had been wilted for a period of several hours regenerated carotene upon being placed in water to restore their turgidity. This was an absolute rather than a relative increase. Under certain optimum conditions carotene in carrots apparently can increase in concentration during storage (124, 125).

A number of investigators have been interested (126 to 130) in the problem of carotene as a source of vitamin A for humans and rats. Consensus of opinion seems to be that carotene is available to the extent of 0 to 40 per cent when fed without fat, but, when given in a solution of fat, the availability increases to levels around 70 to 80 per cent. The amount of carotene available from carrots, and probably most vegetables, seems to be proportional to the amount of destruction of the carrot cell membranes.

ANALYSIS

Evidence continues to be presented that physicochemical estimations of vitamin A in natural oils are not fully reliable (131, 132). Spectrophotometric corrections proposed by Morton & Stubbs (133, 134) have, in general, tended to raise the conversion factor of natural fish liver oils from the value of 1600 ordinarily used in England to approximately 1800. Recently the same authors (135) have re-examined halibut liver oil and determined the relationship between biological potency and ultraviolet absorption. It was found necessary to correct for absorption at 328 $m\mu$ due to constituents other than vitamin A. The conversion factor was found to change from 1570 to 1830 and the average of 1800 is proposed for the general use in converting E-values (Morton-Stubbs corrected) to biological units.

The Sobel-Werbin reagent for the determination of vitamin A has been used in the analysis of human milk (136) and in vitamin A concentrates (137). The reagent, glycerol dichlorohydrin, can be most easily activated by the addition of 2 per cent concentrated hydrochloric acid (137) and should be used soon after activation, since the stability of the chromophore decreases on storage. Mild criticism has been leveled by Wall (138) against this reagent for the determination of vitamin A in fortified poultry feeds. An empirical correction formula is necessary and the method is not applicable to free vitamin A alcohol. The interesting suggestion has been made (139) that by mixing this reagent with the Carr-Price $SbCl_3$ reagent a color with improved intensity and permanence can be obtained.

Using the Carr-Price reagent, Hochberg (140) has improved the chemical analysis of vitamin A in milk, Parrish and co-workers (141) have studied the vitamin A and carotenoids in the blood serum of dairy cattle, and Narod & Verhagen (142) have analyzed

mixed feeds for vitamin A content. In an effort to improve the objectionable rapid fading of color in the Carr-Price reaction, Koch & Kaplan (143) have evolved a spectrophotometric measurement of the blue color which can be made simultaneously on both standard and unknown, thereby cancelling errors due to fading.

Improvements in the chemical estimation of carotene in vegetable tissues have been made by several investigators (144 to 150).

French workers (151, 152) continue to assert that there are substances in some foodstuffs which have vitamin A potency and can be detected by bioassay but which do not give chemical and physical tests for either vitamin A or carotene.

CLINICAL

It is generally accepted that there are substances in certain fish liver oils which have an antihypertensive effect when given to animals or humans. However, these fish liver oils cannot yet be assayed for antihypertensive potency, and large quantities are needed to produce the effect, so that little therapeutic use has been made of them, except in France and Italy (153 to 156). They have been injected intramuscularly several times weekly, 20,000 to 40,000 I.U. of vitamin A per injection. Results have been good in about 60 per cent of the cases. It is believed that cholinesterase activity is repressed or that hypertensive factors resulting from improper metabolism of amino acids are neutralized.

A variety of skin diseases, known by many different names, have been related to vitamin A deficiency. All of these dermatological abnormalities, atypical lichen planus, keratosis seborrheica, keratosis senilis, pityriasis, leucoplakia, xeroderma, lupus vulgaris, and acanthosis nigricans, have the common symptom of hyperkeratotic tissue in the epidermis. This is typical of vitamin A deficiency in animals and it seems logical to treat such cutaneous abnormalities with vitamin A which has been done with unquestionably beneficial results (157 to 166). In many cases, the blood vitamin A and carotene levels of the afflicted patients were below normal. Administration of vitamin A was necessary in large doses, 200,000 to 400,000 units daily, over considerable periods of time, in some instances 19 months, before clinical improvement was brought about. Topical application of vitamin A was also reported effective. The consensus of opinion seemed to be that vitamin A deficiency was the cause of the skin abnormality even

in those cases where the diets were analyzed and a perfectly adequate intake of vitamin A and carotene was found. Liver damage in many of the experimental subjects probably contributed to this induced vitamin A deficiency.

A very interesting skin condition, psoriasis vulgaris, has been studied by Hoffmann *et al.* (167) in relation to carotene and vitamin A metabolism. In this disease there is insufficient keratin produced for the protection of the epidermis against injury. Since vitamin A deficiency results in an excess of keratin production, the patients were placed on a diet supplying only 100 I.U. or less daily for a period of about six months. The plasma carotenoid content dropped to extremely low levels after a week, but the plasma vitamin A levels maintained their initial value throughout the entire experimental period. Seven of 11 patients showed complete clearing of the lesions. These relapsed when normal, unrestricted diets were resumed, or when the restricted diet was supplemented with carotene. While this dietary restriction is not a practical therapeutic regime, it does afford an opportunity for further investigation of the metabolic aspects of psoriasis. Lovino (168) treated rhinitis with vitamin A on the assumption that any abnormality of the epithelial cells of the mucous membrane of the body may be related to a deficiency of vitamin A. Again, large doses were given daily with slow response. A close relationship was shown (169, 170) between low blood levels of vitamin A and carotene and also poor vitamin A absorption curves with high threshold values and delayed recovery times for both rods and cones in dark adaptation tests. In general, the clinical results indicate that drastic measures, such as large doses, synergists, extended treatment, etc., are needed to overcome the tendency for the blood to supply insufficient vitamin A to affected parts of the body.

The amount of vitamin A in the liver of humans is usually a good indication of the vitamin A status of the body. An exception is acute liver disease, in which case the amount of vitamin A in the liver can be quite normal or even above normal, and still the individual may suffer from vitamin A deficiency because of inability to mobilize vitamin A from storage. Liver concentrations of vitamin A in normal humans have been reported for Egypt, 120 units of vitamin A per gm. (171); for England, 300 units per gm. (172); Scotland, 500 units per gm. (173); and Africa, 800 units per gm. (174).

Several investigators concerned themselves with the levels of vitamin A and of carotene in human blood plasma as an index of vitamin A status of the body (175 to 177). In general, the levels of vitamin A and carotene in the blood are relatively constant for each individual and do not reflect the intake of carotene and vitamin A in food. Popper and co-workers (178) studied the variations in free and esterified vitamin A in the blood of normal and pathological humans. Vitamin A alcohol, more than vitamin A esters, is significantly decreased in malnutrition, infections, hepatic and wasting diseases apparently due to reduced storage in, or release from, the liver. The vitamin A alcohol level, therefore, rather than total vitamin A, is recommended as an index of hepatic vitamin A storage and of vitamin A nutriture. Eden & Sellers (179) make the interesting observation on sheep, rats, and cows that vitamin A reaches the blood stream principally by way of the lymph system. In no case was the vitamin A concentration in the portal blood higher than in the systemic circulation.

In studies on middle-aged and old people (180, 181) it was found that, in general, vitamin A levels are below normal. Van Bruggen & Straumfjord (182) supplemented diets of 36 patients with 100,000 units of vitamin A daily for three years and compared the carotene, vitamin A, vitamin E, cholesterol, phospholipid, and vitamin C levels in these patients with the same in the blood of 36 unsupplemented patients. After this period, the vitamin A level was only 125 per cent greater than at the start of the experiment, and six months after supplementation had been discontinued the level of vitamin A had almost returned to the original. Also, for some unexplained reason, vitamin E and cholesterol and phospholipid levels of the blood were increased because of the vitamin A supplementation. Six months after therapy had ended, the levels of these compounds had decreased to their original values. Neither carotene nor vitamin C levels were affected.

Hypervitaminosis.—Several interesting reports have been made concerning the physiological effects of massive doses of vitamin A in animals and toxic effects of vitamin A in humans. Young rabbits, given 10,000 units or more of vitamin A daily for two months, showed thickening of the cornea and other classical signs of hypervitaminosis A (183). Maddock, Wolbach & Jensen (184) have reported that massive hemorrhages leading to death can occur in rats receiving toxic doses of vitamin A only if the diet is also

deficient in vitamin D and phosphorus. The hemorrhages are definitely related to hypoprothrombinemia.

Toomey (185) observed classical vitamin A toxicity in a two-year-old infant who had received 500,000 units of vitamin A daily for about three months. The vitamin A concentration in the plasma reached a level of 400 μg . per 100 ml. Enlargement of the liver, fragility of the bones, and increased levels of serum phosphatase were all observed. Dickey & Bradley (186) observed the same symptoms in a three-year-old child who had received 400,000 units of vitamin A daily for at least a year and a half.

VITAMIN D

CHEMISTRY

Velluz and co-workers (187) postulated the existence of a new vitamin D_2 precursor convertible into calciferol by heat, since irradiated ergosterol preparations increased in potency after being refluxed in benzene. The configuration of the vitamin D_2 molecule is slightly different from that usually pictured; Crowfoot & Dunitz (188), by a detailed x-ray analysis of a crystalline derivative of calciferol, showed that carbons 5, 6, 7, and 8 lie in a straight chain rather than as a coiled ring (ring B). Three different syntheses of 7-dehydrocholesterol, provitamin D_3 , have been described during the year (189, 190, 191).

Great strides have been made in the analytical determination of vitamin D by physiocochemical methods. Ewing and co-workers (192) have adapted their vitamin D_3 method for use on concentrates containing vitamin D_2 . The oil sample containing at least 50,000 units per gm. is saponified, chromatographed to remove interfering materials, and the filtrate measured for ultraviolet absorption at 265 $\text{m}\mu$. The average deviation from the bioassay values was only 14.9 per cent on 49 samples. They also (192) made use of the SbCl_3 reaction on the unsaponifiable fraction of oils. Absorption is measured at 500 $\text{m}\mu$ three minutes after adding the SbCl_3 . Average deviations from bioassay values were approximately 16 per cent. Rouir and co-workers (193, 194, 195) have adapted the Sobel-Werbin reagent for determining vitamin D_2 in alcohol solutions or in high potency pharmaceuticals. It is highly specific for vitamin D_2 after removal of tachysterol by reaction with maleic or citraconic anhydride. As little as 1 μg . of vitamin D_2 per ml.

solution is detectable. The reagent has been used for vitamin D₃ by Campbell (196) who found a straight-line relationship between absorption and vitamin D₃ concentration between the limits of 2 and 25 µg. of vitamin D₃ per ml. Bioassays confirmed the usefulness of this chemical method for high potency oils, but further refinement will be necessary before it can be applied to low potency oils.

PHYSIOLOGY

Vitamin D increases the utilization of phytin phosphorus but this is apparently not mediated through phytase formation since intestinal phytase activity in rickets is essentially normal (197). The antirachitic action of dietary citrate is explained by Hurni (198) as due to the formation in the intestinal tract of a non-ionizable calcium citrate compound which is well absorbed from the gut but is poorly excreted by the kidney. Thus, a positive calcium balance is obtained. The antirachitic effect of coconut oil has been confirmed (199) and is attributed to a decreased loss of both phosphorus and calcium in the feces.

Casella and co-workers (200) have measured the respiratory quotients of rachitic rats receiving various levels of vitamin D. They found high values (above 0.9) in all groups, indicating no impairment of carbohydrate metabolism in vitamin D deficiency.

Vitamin D₂ has no estrogenic activity (201), but there seems to be a relationship between vitamin D and the sex hormones. The male sex hormone is slightly rachitogenic whereas the female hormone is antirachitic (202). Vitamin D₂, in daily doses of 5 mg., and stilbesterol have been reported by Nasio (203) to protect dogs against peptic ulcers induced by cinchophen.

Giroux (204) found that large doses of vitamin D₂ prolong the resistance of guinea pigs to infection by the tubercle bacillus. However, after the disease was established, vitamin D₂ was neither bacteriostatic nor bacteriocidal. Raab (205) preferred to use unactivated ergosterol which shows the same antitubercular activity as vitamin D₂, without the possibility of toxicity.

Vitamin C absorption and excretion have been studied (206) in 36 rachitic children before and after treatment with vitamin D. The results, while not of obvious significance, are nonetheless definite. Urinary excretion of vitamin C following a 150 mg. injection was 50 to 67 per cent less after the vitamin D treatment.

Bioassay.—Various investigators (207 to 212) have been

concerned with the accuracy and precision of vitamin D bioassays of foods and pharmaceuticals by standard (U.S.P., A.O.A.C., and B.S.I.) procedures. The U.S.P. Reference Standard for vitamin D is variable in potency as shown by bioassay with either rats or chicks against crystalline vitamin D₂ or D₃. A new international standard, represented by crystalline vitamin D₃, seems definitely needed.

DISTRIBUTION

Newlander (213) measured the vitamin D potency of various hays used in the feeding of dairy cattle and found a range from 154 to 1,134 units per lb. Other workers (214) report 213 units for barn-dried hay, 254 units for wilted silage, and 440 units per lb. for field-cured hay.

Vitamin D₂ as well as vitamin D₃ has been identified (215) among the sterols of mussels after ultraviolet irradiation. A report from Argentina (216) that sardines canned in oil average 208 units of vitamin D per 100 gm. is interesting, since canned fish is about the only rich source of vitamin D in human diets.

The level of vitamin D in human milk has long been known to reflect the maternal intake of this vitamin. However, a recent report (217) gives some new and interesting quantitative data. Oral administration of a single dose of 600,000 units of vitamin D increased the vitamin D in the milk of two women to 1,000 units per l. within 24 hr. Even after one month the milk contained 100 units per l., 10 times the original level.

CLINICAL

The National Research Council has revised (218) its Recommended Dietary Allowances only slightly with respect to vitamin D. The requirement during pregnancy and lactation has been changed from 400 to 800 units to 400 units. However, the need for vitamin D in adolescence is now considered to be as great as that in infancy (400 units).

Rickets in infants still appears frequently in pediatric clinics of many U.S. teaching hospitals and, probably to a greater degree, in other institutions (219). Large, single doses (220) of vitamin D, as well as small, regular doses (221), are very effective in rickets. Fifteen milligrams (about 600,000 units) of vitamin D either orally or by injection will keep an infant supplied for approximately four months. Analysis of tissues of an infant given 50 mg. of vitamin D

who died soon after from other causes showed no vitamin D in the muscles, heart or lungs, only a trace in the liver, brain, and kidneys, and relatively large amounts in the subcutaneous tissue.

Although the majority of recent reports relative to vitamin D treatment in tuberculosis has been favorable (222), it has not been generally accepted as standard therapy. Cornbleet (223) administered calciferol, 100,000 to 150,000 units daily, for several months and noted temporary improvement in the symptoms of lupus vulgaris. In addition, injections of streptomycin used in conjunction with the vitamin D were so effective as to suggest a synergistic action. Cysteine appears to be antagonistic to vitamin D (224). Daily injection inhibited both the antituberculous and toxic action of the vitamin.

Healed skin lesions in persons with tuberculosis receiving calciferol (225) had a higher calcium content than normal skin, indicating that vitamin D makes calcium more available to lupus tissue.

Four tubercular humans treated with 300 to 500 mg. of non-irradiated ergosterol were reported (205) as showing some clinical improvement after six months. This needs confirmation since nonactivated provitamin D₂ is supposedly not absorbed from the gastrointestinal tract.

Hypervitaminosis.—Recent studies (226 to 231) have emphasized that the safe, daily dose for medication with vitamin D seems to be about 5,000 units per kg. of body weight, and that toxic symptoms which result from larger intakes persist for many months. The dangers involved in using massive doses of vitamin D in the treatment of arthritis apparently outweigh the subjective benefits and this therapy is being used less frequently. Vitamin D injected intramuscularly has been reported (232) to cause no injury, whereas the same dose given orally induced typical symptoms of vitamin D toxicity.

VITAMIN E

CHEMISTRY

The coumarin isomer of α -tocopherol was synthesized by Smith & Boyack (233) and, although it differs only in having a five-member instead of a six-member heterocyclic ring, it possesses a biopotency only one-twentieth that of α -tocopherol. Other advances in the chemistry of vitamin E during the past year are shown by improve-

ments in analytical procedures (234 to 238). Quaife (239) developed a scheme for the analysis of individual tocopherols in mixtures of the α -, β -, γ -, and δ - forms. Yellow nitroso derivatives of the various tocopherols, except α -tocopherol, can be formed and then separated quantitatively by simple chromatography. Colorimetric analyses of the eluates give the individual and combined content of β -, γ -, and δ -tocopherol. Total tocopherols are determined by the Emmerie-Engle method and the α -tocopherol content is calculated by difference.

The well-established stabilizing action of vitamin E on vitamin A *in vitro* (240), as well as the value of synergists such as lecithin (241), has been confirmed.

PHYSIOLOGY

The mechanism whereby vitamin E functions in the body is still unknown. This is also true of some of the other vitamins but in contrast with the others there is no comprehensive, unifying theory explaining the observed physiological activity of vitamin E. Hickman & Harris (242) several years ago proposed a dual role for the tocopherols *in vivo*. First, α -tocopherol exerts a true, specific, vitamin-like function, perhaps as an enzyme component or co-factor. Muscular dystrophy is probably related to this function of vitamin E. Second, the tocopherols exert a secondary, less specific function probably by virtue of their antioxidant action or by participation in oxidation-reduction reactions *in vivo*. The term secondary refers to the specificity and the essentiality of this function and not to its importance. Ceroid pigment formation and deposition in tissues, vitamin A sparing-potency, and many other phenomena are related to this very important function of vitamin E. To these, a third role for vitamin E may now be added. Tocopherols exert a pharmacological action when administered in large amounts which may be generally beneficial or detrimental, depending on the tissue or syndrome under observation. Decreased conversion of carotene or inhibition of vitamin A storage is brought about by administering massive doses of vitamin E. Conversely, desirable effects which seem due to improved circulatory function are induced by large doses of tocopherols. These theoretical roles are described merely as a guide in evaluating the following discussion.

Absorption and excretion.—Recent studies on the absorption

of vitamin E in oil solution from the gastrointestinal tract of rats in which α -tocopherol labelled with radioactive C^{14} was used, indicate that about 80 per cent of the vitamin is excreted (243). This should not be surprising in view of Engel's findings (244). Forty per cent of vitamin E from wheat germ oil and 87 per cent from dried grass were excreted in the feces. The magnitude of loss of ingested tocopherols by fecal excretion (40 to 87 per cent of physiological-sized doses), plus the quantity probably destroyed by oxidation in the gastrointestinal tract, emphasize the need for improving absorption of vitamin E.

Interrelationships with enzymes.—The oxidation of linoleic acid by crystalline lipoxidase (245, 246) is inhibited by tocopherols. Hickman (247) points out that this is a selective inhibition in which the desired, enzymatic oxidation of linoleic acid is permitted but the undesired autoxidation by linoleic peroxides is prevented. Tocopheryl phosphate plays a more direct part in the diphosphopyridine nucleotidase system (248, 249), in which the effect is an inhibition. Hess & Viollier (250) find that plasma lipase and cholinesterase activities are markedly reduced in vitamin E deficiency. Tocopheryl phosphate, according to Zierler and co-workers (251), has pronounced antithrombic and antiproteolytic activity *in vitro*. Hummel & Basinski (252) have determined the oxygen consumption of muscle strips taken from dystrophic rabbits. The oxygen uptake was roughly double that of normal strips. *In vitro* addition of α -tocopheryl phosphate had no significant influence on the oxygen uptake of either normal or dystrophic muscle strips or slices. Hummel (253) also reported that in both dystrophic hamsters and guinea pigs, muscle adenosinetriphosphatase activity was reduced. α -Tocopheryl phosphate inhibits (254) thyroxine-induced metamorphosis of tadpoles by some catalytic mechanism. Neither pure tocopherol nor tocopheryl acetate shows this activity.

Puig Muset and García-Valdecasas (255) find that normal rats treated with vitamin E show a higher resistance against pure oxygen than untreated controls. The mortality was lower, weight loss was less, and the microscopic lesions were less severe. Previous work (256) indicates that vitamin E-treated animals are also protected against low oxygen tensions.

Antioxidant activity.—Commercial and laboratory practices in the United States for stabilization of fats, including the use of tocopherol for this purpose, have been well summarized by Lund-

berg (257). The E-vitamins, in order of increasing antioxidant effectiveness in lard at 100°C., are α -, β -, γ -, and δ -tocopherols (258). The differences in antioxidant activity are not great, however, and α -tocopherol is probably the most important antioxidant *in vivo* since it is preferentially stored in the body.

Heftmann (259) studied the antioxidant properties of carrot oil and various molecular distillation fractions thereof. Tocopherol was the major antioxidant although carotene acted as an antioxidant in concentrations below 3 mg. per 100 gm. but as a pro-oxidant in higher concentrations. The stability of meat during storage was shown by Major & Watts (260) to be improved by administering tocopherol to the animal prior to slaughter. Rancidity in tissues from rabbits fed or injected with tocopherol was significantly delayed compared with normal controls. Krukovsky (261) found a highly significant correlation between tocopherol content of milk fat and ability of milk to resist oxidized flavors. The stability of fresh-pasteurized milk was improved when its tocopherol content was increased, by feeding, to 3 mg. per 100 gm. of fat and above. Below 2 mg. per 100 gm. of fat, it showed poor keeping quality. He concludes that there is a very practical value in increasing tocopherol content of milk, for stabilization as market milk, and for better nutrition. This effect of vitamin E might explain the differences in the stabilities between winter and summer milks.

Longevity.—Euler in a series of papers (262, 263, 264) concerned with the influence of nutrition on growth, fertility and longevity in rats reports that vitamin E is a very important factor in increasing longevity. McCoord (265) comes to the same conclusion with male rats and believes that the effect of vitamin E in prolonging life is due to some factor other than its sparing action on vitamin A.

Protective action against noxious agents.—Hove (266) found that α -tocopherol protects against carbon tetrachloride poisoning. Rats on a 10 per cent casein diet, injected intraperitoneally with carbon tetrachloride at a level of 2 ml. per kg. of body weight, showed a 90 to 100 per cent mortality within 48 hr. Supplementation with 1 mg. of α -tocopherol daily reduced this mortality to less than 20 per cent. γ -Tocopherol had only slight activity at this level. The effect could be duplicated by methionine and certain purines such as xanthine, and also by increasing the casein level in the diet. It seems very likely that this protective action of tocopherol may be

of some practical importance. Hove proposes it as the basis for a vitamin E bioassay procedure, and Rao (267) suggests it may be of clinical significance in preventing massive necrosis of the liver. Massive hepatic necrosis in the rat, which is similar to that in man, can be prevented by vitamin E supplementation (268).

Vitamin E also protects against alloxan injury in rats as shown by prolonged life and increased ability to store vitamin A (269). György & Rose (270) have made the observation that the toxicological action of alloxan is twofold, direct injury to pancreatic cells leading to diabetes and also red blood cell hemolysis leading to hemoglobinuria and death. Vitamin E is protective only against the latter action.

Jaffe (271), by feeding methylcholanthrene to rats, produced tumors in 40.7 per cent of his experimental animals. However, when vitamin E was given as a supplement, tumor incidence was only 16.6 per cent. Tocopherols may be a factor in tumor metabolism as an antioxidant for unsaturated fatty acids (272). This would lend credence to Park's theory of carcinogenesis (273), in which he postulates intracellular hydroperoxides. He further suggests that this type of free-radical, branched-chain oxidation reaction might be started in tissues by peroxidation of a carcinogenic hydrocarbon. Compounds which would break the free-radical oxidation chain should inhibit cancer by retarding the oxidation.

Reproduction.—Vitamin E, although no longer considered a fertility vitamin exclusively, is an important factor in the reproductive processes of many species of animal, both male and female.

In the male rabbit, vitamin E deficiency shows up as well-developed lesions in the reproductive system, similar to those described in vitamin E-deficient rats (274), although dystrophy of straited muscles occurs earlier and often causes death.

In the male rat, increased work intensifies degeneration of the testes caused by vitamin E deficiency, according to Kokas and co-workers (275). Apparently there is no decreased androgen production by the testes of vitamin E-deficient rats as judged by the response of smooth muscle to stimulating drugs (276).

Blahak (277) treated a series of 35 sterile men with 50 mg. of vitamin E daily for three months and found evidence of improved spermatogenesis. Reports (278) at a conference on human infertility were contradictory relative to the value of vitamin E. Fifty cases with abnormally low sperm counts were treated with 60 mg. of

vitamin E daily. There was an increase in density of spermatozoa in 23, and in 18 an improvement in motility and viability, and in 17 an increase in volume of more than 0.5 ml. A lower percentage of abnormal sperm occurred in all cases showing improvement. However, other reports at the same meeting indicated that no apparent beneficial response was obtained in sterile males, although in these case relatively small doses of tocopherols were given. Jungck and co-workers (279) found normal blood levels of vitamin E in instances of male sterility.

In the female rat, lack of vitamin E not only induces fetal resorption but more fundamentally the presence of this vitamin is essential for the ability to become pregnant. Kaunitz and co-workers (280, 281) find that implantation of the fertilized ovum in the wall of the uterus is dependent on vitamin E in both young and old rats. The requirements for implantation are only a fraction of those necessary for normal gestation and lactation. It is still uncertain whether implantation failure is caused by ovarian or uterine dysfunction, by disturbances in fertilization, or by abnormal passage of the ovum from the ovaries to the uterus (281).

Menschick (282) reports that vitamin E influences fat metabolism in the ovary of mice. Neutral fat deposition increases while the formation of other lipids is inhibited.

Athanassiou reports, in a series of papers (283, 284, 285), on the significance of vitamin E in reproduction in humans. The placenta averaged 0.75 mg. of vitamin E per 100 gm. Also, arterial cord blood was richer than venous cord blood in vitamin E by two to five times. A large proportion of women (58 of 90) who miscarried had very low blood levels, 0.2 to 0.3 mg. of vitamin E per 100 ml. However, other investigators (286, 287) find no difference in the tocopherol content of the blood of normal, pregnant, and aborting women.

Ferguson (288) has studied 66 patients with menopausal syndromes over a period of 18 months. α -Tocopherol was given orally and in some cases by injection. Sixty of the patients obtained practically complete relief from their flushes and other symptoms which led the author to conclude that vitamin E has definitely demonstrated its effectiveness in menopausal syndrome therapy.

A review by Pappenheimer (289) deals with the pathological changes which occur in the body due to vitamin E deficiency and, also, the reparative processes which occur when vitamin E is given

curatively. A particularly interesting point is that muscular dystrophy has been found to develop late in intra-uterine life. The mother seems to remain healthy, but the young may be born dead or survive only a short time. That dystrophic infants may be born of an apparently healthy mother raises the interesting question whether nonapparent maternal deficiencies may be operative in certain kindred human disorders.

Some time ago α -tocopherol was described (290) as having the same effect as estrogens in the castrated rabbit. More recent reports (291) also indicate that synthetic vitamin E, either as tocopheryl acetate or as the free tocopherol, given subcutaneously to young rats, has estrogenic activity. Results were negative when the vitamin was given subcutaneously to adult rats or when natural tocopherols were used.

Lactation.—Squibb *et al.* (292) report results showing that soybean products, probably because of their vitamin E content, increase the utilization of vitamin A in the cow as measured by increased blood plasma levels.

A daily supplement of more than 333 mg. of tocopherol is necessary to increase the amount of vitamin E secreted in the milk (293). γ -Tocopherol is less efficiently transferred to the milk than is α -tocopherol. Cows, receiving tocopherol supplements (294) during the later stages of gestation, produce colostrum milk very rich in vitamin E, and the increased vitamin E output is maintained even after the colostrum period. Later milk, which is used for human consumption, is from one-tenth to one-twelfth the potency of colostrum in vitamin E. Also, cows' milk contains only 60 μ g. of tocopherol per 100 ml. whereas human milk contains 940 μ g. per 100 ml. Human colostrum is also very high in tocopherol ranging from 2,600 to 3,300 μ g. per 100 ml. (295). According to Neuweiler (296), supplementing diets of lactating mothers with vitamin E causes an increase in tocopherol content of only those milks which were originally subnormal. The concentration apparently never exceeds physiological limits which Neuweiler considers from 250 to 3,000 μ g. per 100 ml. The corresponding range for cows' milk is from 50 to 150 μ g. per 100 ml.

Gullickson and associates (297) were unable to show that tocopherol supplementation of dairy cows increased the amount of fat produced or the percentage of fat in milk, in agreement with Whiting and co-workers (298) who also observed that tocopherol

supplementation failed to prevent the decrease in butterfat concentration induced by cod liver oil feeding. The tocopherol content of the milk fat and blood plasma was increased by feeding tocopherols but was decreased by feeding cod liver oil. The depressing effect of cod liver oil on the carotene content of milk fat and of blood plasma was counteracted somewhat by feeding tocopherol. Previous experiments (293), in which tocopherol supplementation did increase milk-fat output, were probably conducted with cows more deficient in vitamin E than those discussed above.

Rauramo (299) notes that the elevated blood tocopherol level of women at parturition gradually returns to pre-pregnant levels after about two months.

Muscle.—Willman (300) has reported that vitamin E is the factor lacking in the diet of pregnant ewes which causes them to bear lambs which develop stiff-lamb's disease. This is actually a muscle dystrophy similar to that seen in other species due to vitamin E deficiency. Whiting and co-workers (301) have found further that the incidence of stiff lambs is greater when alfalfa or clover hay is fed than when nonlegume hay is used. The tocopherols in legumes are relatively low in α -tocopherol and, therefore, relatively less potent which probably explains the inferiority of legume hay.

Dystrophy and azoturia observed (302, 303) in horses is similar to stiff-lamb's disease, as are the muscle changes seen in young calves (302, 304), which have been raised from birth on a diet consisting mainly of skim milk.

Weanling pigs, according to Krider (305), grow faster and require less feed per unit gain when alfalfa meal is given at a 4 per cent level. With 10 per cent alfalfa meal gains are more rapid and economical. By inference, their results can be interpreted as due to the vitamin E content of the alfalfa meal supplement.

In the treatment of human muscular dystrophies with vitamin E, Milhorat (306) reports that δ -tocopherol given in doses of 220 mg. daily reduces creatinuria in some of his patients, particularly those with the facial-humoral-scapular type of dystrophy.

Steinberg's original observation (307) that Dupuytren's contracture, a type of fibrositis which yields to vitamin E therapy, has been noted by Parsons (308), who tried vitamin E treatment in a single case with negative results. Another type of fibrositis, Peyronie's disease, has been treated with vitamin E by Scott &

Scardino (309). Twenty-three patients, with long standing contraction of the fibrous connective tissue of the penis, were given vitamin E therapy for 5 to 9 months. Two hundred to 300 mgs. of tocopherol daily was the dose administered. Good results were obtained in 11 cases and fair results in 10 cases. Only two remained unbenefited. Williams (310), in a study of the effect of vitamin E in vague muscular pains of long duration, included a group of diabetics. Almost 50 per cent of these obtained relief from their neuromuscular pains.

Skin.—Burgess & Pritchard (311, 312) have recognized the importance of vitamin E in the diseases classified as collagenoses. Necrobiosis diabetorum lipoidica, lupus erythematosus, atopic dermatitis, dermatomyositis, lichen sclerosis, and granuloma annulare have all shown favorable response to tocopherol therapy, both oral and intramuscular. Sweet (313) failed to confirm the beneficial effect of vitamin E in lupus erythematosus, but McKenna (314) found tocopherols of value in relieving cases of lichen sclerosis atrophicus. Price (315) reported complete confirmation of Burgess' and Pritchard's findings relative to the curative effect of vitamin E in diseases characterized by collagen degeneration.

Heart and vascular system.—Vawter (304), although primarily interested in muscular dystrophy in young, vitamin E-deficient calves, reports that localized heart lesions, as well as generalized skeletal muscle dystrophy, are almost always present. Sixty per cent or more of the calves in certain herds die within 10 to 21 days after birth. Calf losses stopped in a week or 10 days after the cows with calves were placed on green pasture or the diet changed to leafy alfalfa hay. The hearts from the afflicted calves revealed conspicuous yellow or gray foci or streaks of myocardial dystrophy, involving most of the left ventricle. This recalls Gullickson & Calverley's report (316) on cardiac failure in cattle due to vitamin E deficiency.

Martin & Faust (317) find anatomical injury in the hearts of rats and rabbits maintained on a vitamin E-low diet. Butturini (318) describes degenerative lesions in the myocardium, predominantly localized in the left ventricle, in the rat. This was accompanied by a progressive bradycardia, which finally resulted in arrhythmia. Gatz & Houchin (319, 320) report electrocardiographic changes and histological degeneration in the hearts of vitamin E-deficient rabbits. Houchin (321) also observes that such rabbits were more sensitive to posterior pituitary extracts and

more resistant to the toxic effects of cardiac glycosides. Cardiac dilation occurs but the sudden death in rabbits was due principally to myocardial failure.

Experiments by Holman (322), which show prevention of arteritis in dogs by vitamin E, deserve comment. Essentially, the lesion is an acute necrotizing arteritis whose closest human counterparts are periarteritis nodosa and rheumatic arteritis. Lambert (323) reports the quick recovery of dogs and cats from cardiac diseases of various kinds when vitamin E is administered.

Lemley and co-workers (324) found that the mean plasma tocopherol level in patients with heart disease was lower than the normal mean. Also, creatine increases in the blood and the urine of patients with heart disease (325).

Clinical investigations to determine the value of vitamin E in heart disease in humans have been stimulated by the positive nature of most of the experimental work on laboratory and farm animals. To the pioneering work of the Shutes & Vogelsang (326) can be added that of Leinwand (327), Molotchick (328), Pin (329), Valatx (330), and Agadjanian (331). In opposition to these clinical findings are recent negative reports by Baer and co-workers (332), Ball (333), Makinson (334), and Gram & Schmidt (335). The last-mentioned, however, report that 17 of 80 patients were improved and that such improvement must have been in the condition of the blood vessels, especially the coronary vessels. Shute *et al.* (336) and Hickman (337) criticize various aspects of the clinical studies which failed to indicate beneficial effects of massive doses of vitamin E in coronary abnormalities. The American Medical Association (338) suggests that a well-controlled, clinical investigation be initiated to establish the true value of vitamin E therapy in heart disease. The *Lancet* editorially (339) is impressed with the "conclusive evidence from animal experiments that vitamin E participates both in maintaining normal permeability of capillaries and in protecting heart muscle from degeneration."

Vitamin E apparently exerts its pharmacological function in cardiovascular conditions since doses in the range, 400 to 800 mg. daily, are apparently needed. Shute and co-workers (326) emphasize that α -tocopherol is the effective form of vitamin E. Moreover, they rightly contend that pharmaceutical preparations of vitamin E be labelled to show quite plainly the α -tocopherol content, or the International Units, in the product.

In peripheral vascular diseases, Shute and co-workers (340) and

Burgess & Pritchard (341) have studied the effect of vitamin E therapy on ulcers of the extremities and on conditions where thrombi had formed. They report marked beneficial effects. Richtsmeier and co-workers (342) failed to duplicate the work reported by Shute (343) and Vogelsang (344) on the antipurpuric action of vitamin E. However, a recent clinical report (345) indicates that vitamin E in doses of 150 to 200 mg. daily for three to six days stops bleeding in severe and prolonged uterine hemorrhage.

Teeth and gums.—In vitamin E deficiency in rats, dental depigmentation, as well as brown coloration of adipose tissue, depends on the presence of highly unsaturated fatty acids in the ameloblasts and fat cells themselves as well as on the absence of vitamin E. Granados and co-workers (346) believe that pigment deposition in the teeth is inhibited because ferric iron is reduced by unsaturated fatty acids, thus becoming unavailable for pigment formation. Iron concentration in the diet or in the blood plays no part in this phenomenon but vitamin E permits normal pigment formation.

King & Gimson (347) describe lesions of the gum and alveolar bone in hamsters due to tartar formation. Vitamin E-lack may have been a causative factor in this calculus deposition. Goldbach (348) has used vitamin E therapy in patients with parodontopathic conditions. Intramuscular injections of 30 mg. of α -tocopheryl acetate were used, and after three or four injections, severely loosened teeth often became tight without the use of other therapeutic measures. In only three patients, diabetics, has the vitamin E therapy been unsuccessful.

Pancreas.—Diabetic patients usually have creatinuria, according to Caspe (349). A further link between diabetes and vitamin E is reported by Butturini (350), who studied arterial and venous blood sugar levels with and without the administration of vitamin E. In the diabetic, as well as in the normal subject, vitamin E reduced the blood sugar level in the veins, without influencing the arterial blood sugar concentration. Tocopherol apparently enhanced the effect of insulin and in cases of mild diabetes could be substituted for insulin. The mechanism suggested by Butturini is that vitamin E improves the utilization of carbohydrates at the site of muscle fibers. Vogelsang and associates (351) report full confirmation of the beneficial effect of vitamin E in diabetic humans.

Williams (310) and György (270) failed to find improvement in pancreatic function in diabetes but reported alleviation of certain associated symptoms by vitamin E.

Nervous system.—Rubenstein (352) reported on seventeen patients with severe vasomotor symptoms related to the menopause which could not be relieved with barbiturates or other medication except estrogen. Marked reduction in symptoms occurred in 14 of 17 patients when vitamin E was given at a level of 75 mg. per day. Six obtained complete relief. Michael & Ruggles (353) treated 35 subjects randomly chosen in a mental hospital with 50 mg. of tocopherols daily over periods ranging from 7 to 121 days. Two were completely relieved of their nervous symptoms, five showed definite improvement, fourteen showed increased psychomotor activity, and fourteen showed no change. All of the four patients with menopausal symptoms reported complete or significant relief. The authors conclude that vitamin E is of some value in the conservative management of depressed mental patients, particularly those for whom shock therapy is contraindicated.

MISCELLANEOUS

Much research must be done to determine the effects of large doses of vitamin E in animals including man. In heart disease and in diabetes, vitamin E in large doses probably exerts a beneficial pharmacological effect. Conversely, relatively high levels of tocopherol supplementation in rats may interfere with carotene conversion or with vitamin A deposition in the liver (45).

The place of vitamin E in human nutrition where relatively small but regular intakes of tocopherol are involved is a problem for the food scientist as much as for the physician. Oser (354) ably presents this point of view.

Use of nitrogen trichloride (agene) treatment of flour was discontinued in Germany in 1945 on the recommendation of Kuhn (355) because about 80 per cent of the vitamin E present in flour is destroyed by this treatment.

Recent work (356) confirms the greater effectiveness of natural *d*, α -tocopherol compared with synthetic *dl*, α -tocopherol in the standard antisterility test on rats. The natural α -tocopherol is 36 per cent more potent than the synthetic form. Also, esterification of α -tocopherol markedly increases its biological activity, probably by increasing its resistance to oxidative destruction in the gut prior

to absorption. α -Tocopheryl acetate, succinate or probably any simple ester, shows about 62 per cent more potency than an equivalent molecular quantity of α -tocopherol in the free form (356).

VITAMIN K

A colorimetric procedure proposed (357) for the semiquantitative determination of vitamin K consists of reacting the vitamin with 2,4-dinitrophenylhydrazine, and measuring the green hydrazone colorimetrically after extraction with amyl alcohol.

Quick & Stefanini (358) have devised a simple vitamin K-deficient diet which produces a marked and regular hypoprothrombinemia in newly hatched chicks. Menadione, 2-methyl-1, 4-naphthoquinone, injected intravenously, increased the prothrombin level of deficient chicks slowly at first, then more rapidly until the normal level was reached after four hours. Quantities greater than the minimal effective dose did not influence the speed of recovery of prothrombin activity. Excessive intakes of vitamin A, such as cause hypoprothrombinemia in rats, had no effect on the prothrombin level of chicks receiving minimal amounts of vitamin K.

Dam (359) tested Quick's concept that the relatively stable component of prothrombin consists of two factors, one poisoned by dicumarol, the other inactivated by vitamin K deficiency. Plasma from vitamin K-deficient chicks mixed with plasma from chicks poisoned with dicumarol failed to clot normally. Apparently the same component was lacking in the two kinds of plasma. Recently, Quick (358) has reported that vitamin K counteracts the hypoprothrombinemic effect of dicumarol and that vitamin K₁ and menadione are equipotent in this respect.

Glavind and co-workers (360) have found that some samples of saliva contain vitamin K which may be significant in view of the protective action of this vitamin against dental decay. The role of vitamin K in bacterial metabolism has not been determined, but since this vitamin is present in so many microorganisms, it seems probable that it has nutritional importance. Iland (361) studied the requirement of *Mycobacterium tuberculosis* for vitamin K and vitamin K-like compounds. Vitamin K apparently was not necessary and, in fact, it seemed to inhibit the growth of this organism. Tishler & Sampson (362) isolated a compound from the autolyzed cells of a spore-forming soil organism, *Bacillus brevis*, which proved to be vitamin K₂.

Vitamin K₆, 2-methyl-4-amino-1-naphtholhydrochloride, inhibits the growth of fungi and the fermentation of yeast (363). At concentrations of 100 parts per million, it prevents the aerobic development of *Penicillium notatum* (364). The antibiotic effect of certain compounds can be suppressed by vitamin K (365); conversely, the antibiotic action of vitamin K and derivatives upon a variety of microbiological species has also been reported (366).

Thayer (367) has reviewed the relation of vitamin K to the blood clotting process and hemorrhage. Hemorrhages noted in hypervitaminosis A are due to hypoprothrombinemia and vitamin K will prevent them. However, bone fragility which occurs also in hypervitaminosis A is in no way affected by vitamin K (368). Hypoprothrombinemia due to injury of some liver process, e.g., by the intravenous injection of pyramidone, can be corrected by vitamin K (369). However, certain other injuries, such as cancer which causes the liver to lose its ability to form prothrombin cannot be relieved or repaired by vitamin K treatment (370). Menadi-one has an inhibiting effect on the cholinesterase of red blood cells (371).

Large doses of vitamin K can also induce hyperprothrombinemia which disappears within 24 to 48 hr. provided the patient has normal liver function (372).

OTHER FAT-SOLUBLE FACTORS

ANTISTIFFNESS FACTOR

A patent (373) issued to Van Wagtenonk discloses a process for obtaining the antistiffness factor from sugar cane or beets. Simple extraction of cane juice with ether yields a concentrate of approximately one million units per gram. A 500-fold concentration can be effected by selective adsorption, molecular distillation, distribution between immiscible solvents, etc. Other workers (374) are pessimistic concerning further advances until an assay procedure is developed with more precision than the current wrist-stiffness test in guinea pigs.

BUTTER-FAT FACTORS

Jack & Hinshaw (375) and Nath *et al.* (376) described experiments in which certain butter-fat fractions exerted growth responses when fed to young rats greater than that of the original

milk-fat. The fractions were prepared by fractional crystallization or precipitation from acetone or pentane at low temperatures.

The reputed vitamin activity of vaccenic acid has been carefully investigated by Deuel and co-workers (377) and by Euler (378). Both groups report that this fatty acid plays no specific role in the growth of the rat.

VITAMIN F (ESSENTIAL UNSATURATED FATTY ACIDS)

Linoleic acid was found by Fraenkel (379) to be necessary for the emergence of the moth *Ephestia* and for normal formation of wing scales. Arachidonic acid had no effect on wing development but was needed for growth of the larva. Certain moths apparently can synthesize linoleic acid, others cannot. Foster & Wynne (380) have shown that linolenic acid in particular inhibits germination of the spores of *Clostridium botulinum*. Linoleic and oleic acids have some activity while stearic acid is completely inactive.

Rusch & Miller (272) have studied the autoxidation of linoleic acid in systems which also contained carcinogenic aminoazo dyes. The carcinogens were destroyed and it may not be too far-fetched to picture the oxidation of linoleic acid *in vivo* as performing a similar protective function against carcinogenic compounds.

VITAMINS L

Vitamin L₂, one of the lactation vitamins, has been reported (381) in both yeast and liver. It can be extracted from the former, but not the latter, with alcoholic acetic acid. Apparently, vitamin L₂ occurs in liver in a firmly bound, relatively insoluble form.

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WATER SOLUBLE VITAMINS¹

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INTRODUCTION

So many articles in the field of the vitamin B complex were published during 1948 that it is difficult to encompass the subject within a single chapter. Not only does the number of investigations of each vitamin continue to increase, but the number of the vitamins themselves increases from year to year. Considerations of space have made it impossible for the authors of this review to include a discussion of many important scientific contributions.

ASCORBIC ACID

Ascorbic acid in foods and tissues.—A large amount of work has continued on the changes in ascorbic acid and its derivatives occurring during storage of plant tissues. Comparisons between the dye reduction and the dinitrophenylhydrazine methods have shown that the latter does not truly measure the loss in biological activity that occurs during storage. Hartzler (1) found that analysis of fresh guava fruit by the two methods yielded the same results but on stored samples of guava juice the dinitrophenylhydrazine method yielded higher values than the dye reduction. Biological assay by excretion methods with human subjects yielded values in accordance with the dye titrations. It appears probable that the biologically inactive compound which is measured by the dinitrophenylhydrazine method is diketo-L-gulonic acid. This compound, which is the free acid of dehydroascorbic acid, the corresponding lactone, is biologically inactive and reacts with dinitrophenylhydrazine.

A new modification of the dinitrophenylhydrazine method has been proposed by Roe *et al.* (2) which overcomes the above mentioned objection, by permitting the measurement of diketo-L-gulonic acid as well as ascorbic acid and dehydroascorbic acid. Ascorbic acid does not react with dinitrophenylhydrazine while diketo-L-gulonic and dehydroascorbic acids do. Diketo-L-gulonic acid is determined by the dinitrophenylhydrazine method after the

¹ This review covers the period from December, 1947 to December, 1948.

reduction of dehydroascorbic acid to ascorbic acid with hydrogen sulfide. Total diketo-L-gulonic acid and dehydroascorbic acid are estimated by reacting with dinitrophenylhydrazine. Total diketo-L-gulonic acid, dehydroascorbic acid, and ascorbic acid are measured by the dinitrophenylhydrazine method after oxidation with bromine which converts the ascorbic acid into dehydroascorbic acid.

This method of analysis has been applied to animal and plant tissues by Mills *et al.* (3). Fresh plant tissues were found to contain small amounts of dehydroascorbic acid and diketo-L-gulonic acid. Frozen foodstuffs contained from 0 to 75 per cent of the total ascorbic acid derivatives in the form of diketo-L-gulonic acid, while the corresponding value for dehydrated foods ranged from 0 to 32 per cent. These data explain some of the discrepancies observed in the past between assays by the dye reduction and the dinitrophenylhydrazine methods. Diketo-L-gulonic acid would have appeared as dehydroascorbic acid in the original dinitrophenylhydrazine method, but since it cannot be reduced to ascorbic acid by hydrogen sulfide it would not be estimated by the dye reduction procedure. Bedford & McGregor (4) investigated the changes in ascorbic and dehydroascorbic acids during storage of foods in the frozen state. In the fresh products from 4 to 13 per cent of the ascorbic acid was present as the dehydro- form which was measured by the dinitrophenylhydrazine method, so that presumably diketo-L-gulonic acid was included in this value. After frozen storage for six months, the percentage of dehydroascorbic acid represented 22 to 94 per cent.

A comparison was made of the dye titration and the dinitrophenylhydrazine method for measuring destruction of ascorbic and dehydroascorbic acid in pure solution [Guild *et al.* (5)]. Ascorbic acid was found to be stable in solutions of oxalic acid and metaphosphoric acid by both methods. Dehydroascorbic acid decreased by 80 per cent in 10 days in both these stabilizing solutions when measured by the dye titration but remained constant according to the dinitrophenylhydrazine method. Neither solution prevented the conversion of dehydroascorbic acid to a derivative which could not be estimated by hydrogen sulfide reduction followed by dye titration but which still reacted with dinitrophenylhydrazine. On the basis of the work of Mills *et al.* (3), it would seem probable that this derivative is diketo-L-gulonic acid.

Metabolism of ascorbic acid.—The role of ascorbic acid in tyrosine metabolism has received attention. Sealock & Silberstein (6) had previously observed that guinea pigs maintained on an ascorbic acid-deficient diet exhibited a faulty tyrosine metabolism in which they were apparently unable to break open the benzene ring and excreted large amounts of partially oxidized products such as homogentisic acid, *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyllactic acid. Administration of ascorbic acid completely prevented the excretion of these metabolites. This effect has been studied recently by Painter & Zilva (7). When 0.5 to 1.0 gm. of tyrosine was given, the excretion of the abnormal metabolites began as early as 24 to 48 hr. after the vitamin was withdrawn from the diet and reached a maximum in four to five days. Thus a disturbance of tyrosine metabolism occurred while the tissues still contained large amounts of ascorbic acid. Three hours after the administration of 0.5 gm. of tyrosine to guinea pigs on a scorbutic diet an amount of hydroxyphenol compounds equivalent to half the tyrosine appeared, mainly in the large intestine. Twenty-four hours later none remained in the tissues or the intestine and, instead, had appeared in the urine.

In scorbutic individuals a compound appears which is capable of producing methemoglobin *in vitro*. This compound was identified by Fishberg (8) as benzoquinone acetic acid in the urine of patients with cyanosis. The compound was also present in the urine of patients who showed decreased excretion of ascorbic acid. When the urinary excretion of ascorbic acid was increased by administering it, the excretion of benzoquinone acetic acid was reduced and the amount of methemoglobin in the blood decreased. Fishberg suggests that benzoquinone acetic acid is formed in the normal catabolism of tyrosine but that its existence is so short that it is chemically undetectable. In scurvy and in rheumatic fever where there is a decreased ascorbic acid excretion, this quinone persists for a longer time owing to an interference with tyrosine metabolism, and benzoquinone acetic acid may be determined chemically in the urine.

A relationship between ascorbic acid and pteroylglutamic acid has been reported. In view of the previously known increased excretion of phenolic compounds in pernicious anemia, Woodruff & Darby (9) tested the effect of pteroylglutamic acid on the metabolism of tyrosine in scorbutic guinea pigs. They found that the

administration of either pteroylglutamic acid or ascorbic acid to guinea pigs on an ascorbic acid-deficient diet containing 5 per cent tyrosine, decreased the excretion of tyrosyl derivatives and keto acids. Johnson & Dana (10) found that the administration of ascorbic acid to surviving rats depleted of pteroylglutamic acid prevented further appearance of chromodacryorrhea, produced a gain in weight, and caused the white cell count to return to normal. No reticulocyte response was observed and the hemoglobin continued to decrease. However, after three weeks on the ascorbic acid, a reticulocyte response was brought about when the rats were given 50 μ g. per day of pteroylglutamic acid. In chicks ascorbic acid has been reported to produce small increases in hemoglobin when given to a diet partially deficient in pteroylglutamic acid [Lepp *et al.* (11)].

An interrelationship between ascorbic acid and vitamin A in the rat was observed by Mayer & Krehl (12). One of the first symptoms in vitamin A deficiency was a depletion of the ascorbic acid reserves as indicated by approximately 50 per cent reduction in adrenal and blood ascorbic acid levels. Symptoms occurred which resembled scurvy including bleeding of the lachrymal glands, swollen gums with evidence of bleeding, and swelling of the joints. The addition of ascorbic acid to the vitamin A-deficient diet doubled the survival time and the average maximum weight, and prevented the appearance of the "scorbutic" symptoms. A marked hypertrophy and hemorrhage of the adrenals have also been observed in vitamin A deficient rats by Mayer & Krehl (13). Similar effects were observed in foxes by Bassett *et al.* (14), who found that the addition of ascorbic acid to a vitamin A-deficient diet prevented the appearance of vitamin A-deficiency symptoms and increased slightly the vitamin A levels in the blood serum and liver.

A relationship between glycine, glutamic acid, and ascorbic acid was reported by Christensen & Lynch (15). They found that the concentrations of these two amino acids in the liver and muscle of scorbutic guinea pigs were reduced to approximately one third their normal level.

An investigation of the effect of protein level of the diets by Samuels (16) showed that the ascorbic acid contents of liver, kidney, muscle, and plasma of the rat were reduced by the feeding of high protein diets. Brain and the adrenals, on the other hand, showed no such decrease.

A number of investigations have been made on factors influencing the excretion of ascorbic acid. Hawthorne & Storvick (17) found that sodium bicarbonate decreased the urinary excretion of ascorbic acid while the ingestion of ammonium chloride increased it. The plasma ascorbic acid values were reduced in both cases, which showed that the decreased excretion produced by sodium bicarbonate was not due to an increased retention of ascorbic acid.

Factors which influence the excretion of and the blood levels of ascorbic acid have been studied. Colby *et al.* (18) found that the daily feeding of 6 gm. of chlorobutanol to sheep produced an increase in plasma ascorbic acid levels. This effect with sheep is similar to that previously observed by others working with different species. Viral infections tended to increase the urinary excretion of ascorbic acid in humans after the ingestion of salicylates (19).

The effect of ascorbic acid and hesperidin methylchalcone in reducing the toxicity of dichlorophenarsine for rats was investigated by Friend & Ivy (20), who found that the combined administration of ascorbic acid and hesperidin methylchalcone with an LD₅₀ dose of dichlorophenarsine decreased the mortality to about 15 per cent. Either of these two protective compounds alone was much less effective.

Somers *et al.* (21) used isolated discs of turnip leaves floated on a mineral nutrient solution to study changes in the ascorbic acid content of plant tissue. This method eliminated the effect of possible translocation within the plant as a factor in producing changes in ascorbic acid content. The ascorbic acid content of the leaf discs decreased in the dark and increased in the light. Carbon dioxide was necessary for the synthesis of ascorbic acid during illumination. A number of publications have appeared on the ascorbic acid contents of foods and the losses occurring during processing and storage (22 to 27).

THIAMINE

A large amount of work during 1948 has dealt with analytical methods for the estimation of thiamine (28 to 33), with the amounts occurring in foods, and with the losses occurring during food processing (34 to 38).

The problem of how to measure thiamine requirements and assess the nutritional status of an individual continues to be of interest (39, 40, 41). Urinary excretion of a vitamin has been used

as an index of tissue saturation and accordingly as a measure of nutritional adequacy. In order to test the validity of this principle, Salcedo *et al.* (42) followed the thiamine concentrations of different tissues and the urinary excretion during the course of thiamine depletion in the rat. All tissues examined, except brain, underwent gradual reductions of thiamine. The thiamine level in the brain, however, was maintained at a constant level during depletion up to a certain point where it abruptly decreased. This point coincided with the attainment of minimum urinary excretion. This experiment supports the view that the establishment of a minimum urinary excretion in clinical experiments can be used to ascertain the point of tissue depletion.

An interesting experiment was reported on the effect of thiamine supplementation on learning using identical twins [Robertson *et al.* (43)]. The diets which both sets of twins received contained from 10 to 40 per cent less thiamine than that recommended by the National Research Council. One twin of each pair received a supplement of 2 mg. of thiamine and the other a placebo. In a short term experiment of 20 weeks the thiamine supplemented group showed a small though just statistically significant superiority over the other. However, when the experiment was continued for 40 weeks with most of the same subjects no differences between groups were observed. Thiamine deficiency was reported by Tuttle *et al.* (44) to decrease the reaction time of women.

Another unusual function of thiamine was observed in relation to body temperature control in rats by Hill & Holtkamp (45). The maternal diet was found to affect the body temperature control of the young when they were placed in a cold room. High levels of manganese, 1 mg. per day, given to the mother rat facilitated this adaption of the young to lower temperatures. Thiamine antagonized this effect of manganese and decreased the percentage of young who were able to attain body temperature control against the cold.

A series of investigations has been made on the effect of a number of vitamin deficiencies on tissue respiration in rats and ducks, [Olson *et al.* (46)]. Thiamine deficiency depressed the utilization of pyruvate by cardiac muscle and the addition of thiamine *in vitro* partially restored pyruvate utilization to normal. Analysis of deficient and normal duck ventricle showed a positive correlation between thiamine content and ability to utilize pyruvate up to a point representing about 2.5 μ g. of thiamine per gm. of fresh tissue.

By means of a new technique for growing calves on purified diets it has become possible to produce a thiamine deficiency in calves [Johnson *et al.* (47)] and to establish that calves require dietary thiamine. Thiamine-deficiency symptoms were general weakness, incoordination of legs, convulsions, and head retraction.

The effect of vitamin deficiency on resistance to infection has been the subject of many investigations. One series of observations, Kearney *et al.* (48), showed that thiamine deficiency in mice decreased the severity of disease after infection with western equine encephalomyelitis, but showed little effect on final mortality rates. Thiamine deficiency has been shown to produce a moderate impairment of antibody response in rats to human erythrocytes [Carter & Axelrod (49)].

RIBOFLAVIN

Assay.—For the determination of riboflavin and its various nucleotides, Burch *et al.* (50) have developed a procedure which is based on the facts that (a) riboflavin phosphate and free riboflavin have the same fluorescence; (b) riboflavin adenine dinucleotide has 14 per cent as much fluorescence as free riboflavin, but can be converted into riboflavin phosphate by hydrolysis with 5 per cent trichloroacetic acid; (c) the distribution coefficients of riboflavin, riboflavin phosphate, and riboflavin adenine dinucleotide between benzyl alcohol and water are 3.8, 0.02, and 0.01. The fluorescence is measured in a specially designed microfluorophotometer, which permits the measurement of 0.2 m μ g. in 0.5 ml. and the determination of these three substances in 50 c.mm. of blood. In 13 presumably adequately-nourished human subjects the average free riboflavin plus riboflavin phosphate content of the serum was 0.8 μ g. per cent, while that for riboflavin adenine dinucleotide was 2.4. The total riboflavin content in these subjects was 252 μ g. per cent for the white cells plus platelets, and 22 μ g. in the red cells. Comparisons of the amounts of these three forms in the blood of rats maintained on various levels of riboflavin showed that the changes in total riboflavin content of serum were due to variations in the free riboflavin level.

A detailed comparison of methods for the determination of riboflavin in urine was made by De Ritter *et al.* (51). A fluorometric method (52) was found to yield consistently good agreement with the microbiological method.

Kornberg *et al.* (53) have developed a new method for micro-

biological assay using *Leuconostoc mesenteroides*. The riboflavin requirement of this organism is about one fiftieth of that of *Lactobacillus casei*, approximately 0.004 μ g. per 10 ml. of medium producing half maximum growth. The higher sensitivity of this microorganism permits greater dilution of the assay sample and consequently decreases the amount of extraneous matter.

Separation of riboflavin nucleotides has been made by paper chromatography. Crammer (54) found R_f values for riboflavin, riboflavin phosphate, and riboflavin adenine dinucleotide of 0.5, 0.09, and 0.11, respectively, with collidine as the solvent. By this technique, he found that rat and rabbit heart, brain, liver, and kidney contained riboflavin adenine dinucleotide but not the other two forms. Rabbit spleen extract contained free riboflavin but not the nucleotides, and added nucleotides were rapidly broken down, suggesting the presence of an enzyme in rabbit spleen which hydrolyzed the riboflavin nucleotides.

The presence of such an enzyme was demonstrated independently in a study of the synthesis of acetylcholine by tissue extracts in which riboflavin adenine dinucleotide is involved [Comline & Whatley (55)]. Spleen extracts were found to yield only small amounts of acetylcholine under conditions which led to a large synthesis when brain or placental extracts were added. The spleen extracts were shown to contain an enzyme which rapidly hydrolyzed the riboflavin adenine dinucleotide.

The phenomenon of apparent increase in riboflavin content of foods during processing was investigated by Watts *et al.* (56). They found that incubation of pork muscle at 45°C. previous to extraction with acid and digestion with clarase produced an increase as high as twofold in the apparent riboflavin content. The action, which appeared to be enzymatic, proceeded during storage at low temperatures and was accelerated during the preliminary stages of cooking.

Riboflavin requirement of various species.—Two groups of workers have shown that the riboflavin requirement of the chicken can be increased and decreased by strain selection [Lamoreux & Hutt (57) and Lerner & Bird (58)].

A new method of calculating the vitamin requirements for growth has been proposed (59). The requirements were calculated by plotting a logarithmic dose-response curve and determining the point at which the regression line drawn through the region of approximately linear response reached maximum growth. The main-

tenance requirement was obtained by extrapolating this regression line back to zero again. By this method Hegsted & Perry (60) found the riboflavin requirements of ducks to be 4 mg. per kg. of diet for maximum growth and 0.90 mg. for "maintenance" at zero growth. The pantothenic acid requirements were similarly found to be 12 and 1 mg. per kg. of diet, respectively, for maximum growth and "maintenance".

Turkeys were found by Richardson, Sherwood & German (61) to develop a leg disorder on two diets containing 3.0 and 3.7 mg. of riboflavin per kg. but not on two diets containing 4.4 and 5.0 mg. per kg.

Excretion of riboflavin.—The riboflavin and thiamine excretion rates in 374 children were investigated by Lameck *et al.* (40). The average hourly fasting excretions varied from 4.3 to 9.7 μ g. per hr. for thiamine and 32 to 55 for riboflavin. In one group of children a period of dietary improvement was followed by an increase in the average fasting excretion rate of riboflavin of from 39 to 48 μ g. per hr.

The efficiency of utilization of riboflavin in foods in women subjects was determined by Everson *et al.* (62) by comparing the increased urinary excretion after administration of approximately equal amounts of riboflavin as the pure material and in the test dose of food. The availability of riboflavin in ice cream was 90 per cent while that for green peas and almonds were 41 and 39 per cent, respectively.

Riboflavin has been shown to have an effect in wound healing. Bosse & Axelrod (63) found that in deficiencies of riboflavin, pyridoxine, and biotin in rats, there was impairment in wound healing which was similar to, though less severe, than that observed in scorbutic guinea pigs. Severe deficiencies of riboflavin and pyridoxine produced a marked impairment of wound healing while biotin deficiency resulted in only a slight delay in healing. Riboflavin deficiency in rats was found by Nelson *et al.* (64) to have an effect on bone growth. In rats maintained on riboflavin-deficient diets the growth of the tibia was retarded and endochondral ossification was impaired and the hematopoietic tissue was eventually replaced by fat.

NICOTINIC ACID

The relationship between the metabolism of nicotinic acid and tryptophane continues to supply the main interest in this field.

The identification of 2-amino-3-hydroxybenzoic acid (3-hydroxyanthranilic acid) as an intermediate in the conversion of tryptophane to nicotinic acid has been made. It had previously been shown by Beadle *et al.* (65) that a "nicotinic acid-less" strain of *Neurospora* which would respond to both nicotinic acid and to tryptophane would also respond to kynurenine. It had also been found by Bonner & Beadle (66) that another "nicotinic acid-less" strain when grown with the minimum amount of nicotinic acid just sufficient for growth would accumulate a substance capable of replacing nicotinic acid for certain strains of *Neurospora*. This substance was identified as 3-hydroxyanthranilic acid by Bonner (67) and by Mitchell & Nyc (68) and synthesized by Nyc & Mitchell (69).

Experiments have been made on the role of 3-hydroxyanthranilic acid in other species. Volcani & Snell (70) found that neither this compound nor kynurenine were capable of replacing nicotinic acid or enhancing the growth of a suboptimal amount of nicotinic acid for *Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Proteus vulgaris*, or *Torula cremaris*.

Hydroxyanthranilic acid can also replace nicotinic acid in the nutrition of the rat. Mitchell *et al.* (71) has obtained a growth response by the addition of 1 mg. of hydroxyanthranilic acid per gm. of diet. Excretion studies made on the same rats by Albert *et al.* (72) showed that this amount of hydroxyanthranilic acid increased the excretion of nicotinic acid and *N'*-methylnicotinamide to the same extent as that produced by an equal amount of tryptophane. Krehl & Bonner (73) found that hydroxyanthranilic acid is approximately as active as tryptophane in producing a growth response in rats. Kynurenine was 0 to 15 per cent as active and kynurenic acid and anthranilic acid were inactive.

Kynurenine seems to be inactive both in promoting growth [Krehl & Bonner (73)] and in increasing the excretion of *N'*-methylnicotinamide [Rosen *et al.* (74)]. Thus it appears that kynurenine is not an intermediate in the conversion of tryptophane to hydroxyanthranilic acid in the rat.

Experiments with isotopic tryptophane have shown that the pyridine ring of nicotinic acid cannot be derived from the pyridine ring of kynurenic acid [Heidelberger *et al.* (75)]. Tryptophane with C¹⁴ in the β -position was fed to rats and kynurenine, kynurenic acid, and *N'*-methylnicotinamide isolated from the urine. As ex-

pected, the kynurenine contained C^{14} in the β -position while the kynurenic acid contained isotopic carbon in the β -position of the pyridine ring (Fig. 1). The *N*'-methylnicotinamide contained no isotopic carbon. This is in accord with the *Neurospora* experiments of Mitchell & Nyc (68), which indicated that kynenic uracid is not an intermediate in the synthesis of nicotinic acid. The pyridine

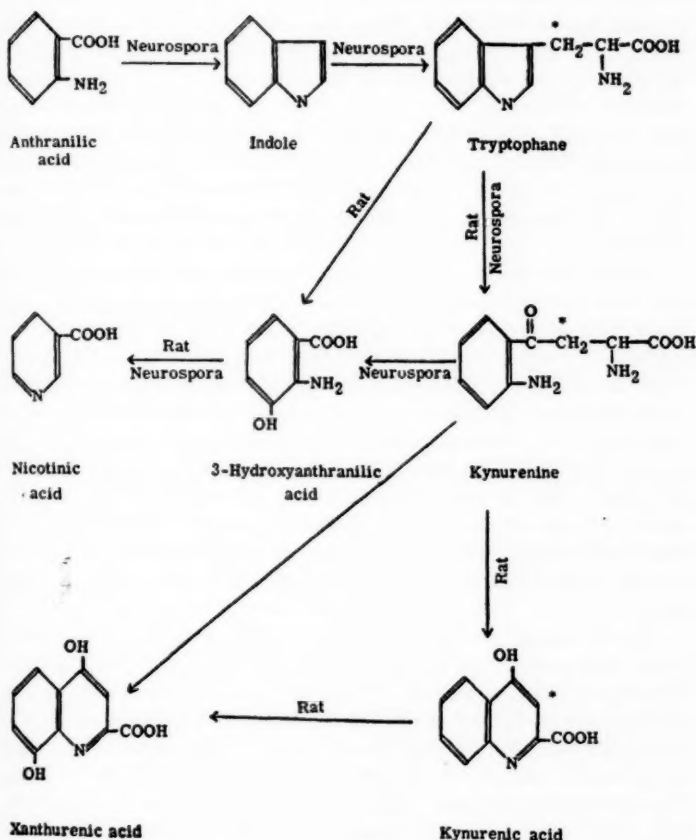


FIG. 1.—Schematic representation of possible conversion reactions between tryptophane, nicotinic acid, and possible intermediates. The position of C^{14} in isotope experiments of Heidelberg *et al.* (75) is shown by *.

ring of nicotinic acid appears to be formed by oxidative rupture of the benzene ring of hydroxyanthranilic acid, loss of a carbon atom, and recondensation with the amino group to form the pyridine ring. Some of the proposed relationships between tryptophane, nicotinic, and intermediate compounds are shown in Fig. 1.

A large number of experiments have been directed toward elucidating the relationship between nicotinic acid, pyridoxine, and tryptophane metabolism. Schweigert & Pearson (76) found that when tryptophane is fed to pyridoxine-deficient rats they have a greatly reduced ability to form nicotinic acid and *N'*-methylnicotinamide. Bell *et al.* (77) observed that pyridoxine did not affect the excretion of nicotinic acid or *N'*-methylnicotinamide on a diet containing 15 per cent of fish protein. However, when tryptophane was added to an otherwise adequate diet containing 15 per cent casein, pyridoxine did increase the excretion of nicotinic acid metabolites.

The formation of xanthurenic acid during pyridoxine deficiency was decreased to one half by omission of nicotinic acid [Spector (78)]. The excretion of *N'*-methylnicotinamide, however, was decreased only slightly in these experiments by pyridoxine deficiency. Further evidence for the necessity of pyridoxine for conversion of tryptophane to nicotinic acid is afforded by the experiments of Ling *et al.* (79). The injection of tryptophane into a rat produced a prompt rise in the diphosphopyridine nucleotide (DPN) level of red blood cells. In pyridoxine-deficient rats no such rise was observed. Pyridoxine was not concerned, however, with the conversion of nicotinic acid to DPN because the rise of DPN in the erythrocytes was the same on normal and pyridoxine-deficient animals.

Pyridoxine is not the only water soluble vitamin which influences the conversion of tryptophane to nicotinic acid [Junqueira & Schweigert (80)]. Omission of either thiamine, riboflavin, or pyridoxine resulted in a diminished excretion of nicotinic acid derivatives. The omission of pantothenic acid had no effect. A marked reduction in urinary excretion of nicotinic acid occurred when succinylsulfathiazole was added and pteroylglutamic acid omitted. The ineffectiveness of the sulfonamide alone to decrease the conversion of tryptophane to nicotinic acid is evidence against the hypothesis that bacterial synthesis is primarily responsible for this conversion.

The production of a nicotinic acid deficiency by the feeding of diets containing large amounts of corn has been credited to a specific or "pellagragenic" factor in corn [Woolley (81, 82)]. Evidence has been accumulating, however, that amino acid imbalance and even the carbohydrate component of the diet can increase the requirement for nicotinic acid (83, 84). The addition of 10 per cent gelatin or certain combinations of arginine, glycine, and alanine to a diet containing 18 per cent crude casein produced a depression of growth in chicks. Growth was restored by the addition of nicotinic acid (83).

The effect of proteins and amino acids on nicotinic acid deficiency in rats was investigated in detail by Henderson *et al.* (84) and Hankes *et al.* (85). On a diet containing 9 per cent casein and using sucrose as the carbohydrate source the addition of 2 per cent of gelatin, of glycine, acid hydrolyzed casein, or an amino acid mixture simulating casein hydrolysate, produced a growth depression which could be prevented by nicotinic acid. However, the effect of the casein hydrolysate was not due solely to the glycine and alanine in the amino acid mixture as the omission of these did not alter the activity of the amino acid mixture in producing a nicotinic acid deficiency. Hankes *et al.* (85) found that DL-threonine or DL-phenylalanine at levels equivalent to that in 2 per cent of hydrolyzed casein aggravated the nicotinic acid-tryptophane deficiency in rats. All other amino acids fed at comparable levels were ineffective. Larger amounts of glycine, or glycine ethyl ester, had a similar effect.

In the human subject, however, gelatin does not appear to affect nicotinic acid metabolism since the ingestion of 20 gm. of gelatin did not affect the urinary excretion of *N'*-methylnicotinamide [Sarett & Goldsmith (86)].

The carbohydrate component of the diet exerts a marked influence on the conversion of tryptophane to nicotinic acid. Schweigert & Pearson (87) found that on diets containing 12 to 18 per cent casein and no nicotinic acid, sucrose gave approximately one half as much growth as that obtained with cerelese. The addition of nicotinic acid to both rations produced a marked growth response with the sucrose diet and a lesser one with the cerelese diet. Thus the differences between these two diets were decreased in the presence of nicotinic acid. The excretion of *N'*-methylnicotinamide, on 18 and 24 per cent casein diets containing cerelese, was three-

fold that on similar diets containing sucrose. Dextrin was also found to increase the excretion of nicotinic acid [Hankes *et al.* (85)].

The question has frequently been raised whether synthesis of nicotinic acid is the result of intestinal fermentation in the animal. The developing chick embryo has been shown to be capable of synthesizing nicotinic acid [Snell & Quarles (88)]. The injection of tryptophane into seven-day-old chick embryos produced a 25 per cent increase in nicotinic acid content over the saline injection controls [Schweigert *et al.* (89)]. However, similar experiments by Kidder & Dewey (90) have failed to confirm this increase produced by the injection of tryptophane. *Drosophila* which was grown under sterile conditions was found to be incapable of converting tryptophane to nicotinic acid [Schultz & Rudkin (91)]. The protozoan, *Tetrahymena geleii*, is unable to convert tryptophane to nicotinic acid [Kidder & Dewey (90)].

The problem of intestinal synthesis of nicotinic acid from tryptophane has been approached by studying the synthesis of nicotinic acid by microorganisms of the intestinal tract. Ellinger & Kader (92) tested the ability of amino acids to stimulate the synthesis of nicotinic acid by *Escherichia coli*. Ornithine produced a marked increase, and arginine and glutamine were less active. Tryptophane, however, had no effect on *E. coli* but did stimulate the synthesis of nicotinic acid by a mixed culture of cecal bacteria.

Isotope experiments by Roth *et al.* (93) have shown that approximately 15 per cent of the carboxyl carbon of nicotinic acid or nicotinamide appears as carbon dioxide within three days when the former two compounds are administered to rats. An isotope experiment has been made which established that *N'*-methylnicotinamide can be derived from dietary nicotinic acid [Hundley & Bond (94)]. Ninety-six per cent of the *N'*-methylnicotinamide was derived from the dietary nicotinic acid when 1 gm. of isotopic nicotinic acid with C^{13} in the carboxyl group was fed per kg. of diet. This experiment indicates that very little was derived from dietary tryptophane. However, the level of nicotinic acid administered was so large that synthesis from tryptophane may have been suppressed.

An interesting series of experiments was made on the methylation of nicotinamide by the rat [Ellinger (95)]. Liver was the only tissue found capable of methylating nicotinamide. No methylation of nicotinic acid occurred, except in the presence of glutamine when

a small amount of methylation occurred when nicotinic acid was added. Using methylation of nicotinamide by liver tissue as an assay method for the determination of nicotinamide, Ellinger (95) studied the formation of nicotinamide. Kidney and brain were the only tissues of those tested which formed nicotinamide from nicotinic acid.

The methyl group of *N'*-methylnicotinamide does not appear to be available for transmethylation. This compound labeled in the methyl group with deuterium did not contribute methyl groups to choline and creatine, and the feeding of *N'*-methylnicotinamide was ineffective in preventing hemorrhagic kidneys in rats on low-choline diets [Keller *et al.* (96)].

The nicotinic acid-tryptophane relationship in the nutrition of the pig has received considerable attention. The requirement of the pig for nicotinic acid had been observed by many workers. Win-trobe *et al.* (97) observed that nicotinic acid deficiency could not be induced in pigs receiving 26 per cent casein. An investigation by Luecke *et al.* (98) showed that the feeding of a diet high in corn containing 5½ per cent casein produced nicotinic acid-deficiency symptoms which consisted of roughening of the hair coat, diarrhea, anorexia, intestinal lesions, and a lowered growth rate. Administration of 30 mg. of nicotinic acid or 1 gm. of tryptophane per day increased the growth rate and the excretion of *N'*-methylnicotinamide. At a 12 per cent level of casein the growth was equal to that obtained with 5 per cent casein plus added tryptophane. This shows that tryptophane is the main limiting amino acid on a 5 per cent casein diet.

Similar experiments were carried out by Powick *et al.* (99, 100) and Burnett *et al.* (101) on the tryptophane and nicotinic acid requirements of pigs on both purified and practical diets.

PYRIDOXINE

The role of pyridoxine in the metabolism of amino acids has been indicated by many experiments. The presence of pyridoxal or pyridoxamine can modify the requirement of microorganisms for certain amino acids. Speck & Pitt (102) observed that pyridoxal or pyridoxamine could replace cystine in the growth of *L. casei*, *L. arabinosus*, or *S. faecalis* at 37°C. but not at 30°C.

Boyd *et al.* (103) found that *Clostridium perfringens* did not need lysine, alanine, aspartic acid, and glycine, provided that py-

ridoxal or pyridoxamine were furnished. Lyman & Kuiken (104) reported that the utilization of D-amino acids by *L. arabinosus* was increased when pyridoxamine was furnished instead of pyridoxine.

Winsten & Eigen (105) used paper chromatography in an elegant method to separate the various pyridoxine derivatives. They located the "spots" of active material by placing the paper strips on an inoculated agar medium deficient in pyridoxine. The R_f values (ratio of the distance the substance has moved to the distance the developing solvent has traveled) for pyridoxamine, pyridoxal, and pyridoxine were 0.18, 0.68, and 0.75, respectively. A solution containing pyridoxal and casein hydrolysate when subjected to chromatography yielded two zones, one of which was identified as pyridoxamine. This conversion amounted to approximately 50 per cent after standing for several days at 0°C. This new application of paper chromatography should prove useful in resolving the different "conjugates" of certain essential growth factors.

An assay method for pyridoxal has been developed by Rabinowitz *et al.* (106). *L. casei*, which responds to pyridoxal but not to pyridoxine or pyridoxamine, was used as the assay organism. In this method, the samples must be sterilized separately from the medium because pyridoxal reacts with amino acids to form pyridoxamine which is inactive. Since pyridoxal phosphate is inactive for *L. casei*, the sample is hydrolyzed at pH 1.8 to yield free pyridoxal.

In contrast to *L. casei*, which cannot utilize pyridoxal phosphate, certain organisms such as *L. helveticus* and *L. acidophilus* require the phosphate of either pyridoxal or pyridoxamine. Pyridoxamine phosphate was three to six times as active as pyridoxal phosphate, and pyridoxal and pyridoxamine were inactive.

Heyl *et al.* (107) prepared a series of pyridoxyl amino acids by catalytic reduction of the Schiff bases formed by the reaction of pyridoxal and amino acids. The compounds were found by Snell & Rabinowitz (108) to be essentially inactive for either growth promotion or inhibition when tested with four different assay organisms, provided that precautions were taken to exclude oxygen during preparation of the samples. In the presence of air, some growth-promoting activity was obtained. The presence of antioxidants prevented the formation of active compounds during autoclaving of the pyridoxyl amino acids in the presence of air.

The role of pyridoxine in protein metabolism of animals has continued to be a subject of interest. It was shown that an increase in the level of protein in the diet of rats elevated the requirement for pyridoxine (109, 110).

The addition of cystine, or methionine, to a pyridoxine-deficient diet containing 15 per cent of casein was found by Cerecedo *et al.* (111) to accelerate the deficiency and to decrease the survival time. An increased requirement for pyridoxine was observed to exist in the toxic condition produced by feeding large amounts of glycine [Pagé & Gingras (112)] or of DL-serine [Fishman & Artom (113)]. A deficiency of riboflavin was found by Porter *et al.* (114) to increase the percentage of tryptophane which was excreted as kynurenic acid, kynurenine, and xanthurenic acid. Thiamine deficiency had only a small effect. The effect of riboflavin in increasing the excretion of tryptophane in the form of these three metabolites fits in with the observation that riboflavin deficiency decreases the conversion of tryptophane to nicotinic acid derivatives as noted by Junqueira & Schweigert (80). An increased urinary excretion of xanthurenic acid following administration of tryptophane to pyridoxine-deficient monkeys was observed by Greenberg & Rinehart (115).

Convulsive seizures in pyridoxine-deficient rats were investigated by Davenport & Davenport (116). Increased brain excitability as measured by a reduction in electroshock threshold was found both in pyridoxine-deficient rats and in the pair-fed controls. Administration of pyridoxine produced a prompt increase in the electroshock threshold in the deficient rats but not in the pair-fed controls. The administration of tryptophane to pyridoxine-deficient animals increased brain excitability. The heightened brain excitability was not the result of increased formation of xanthurenic acid, as the injection of this compound into normal rats had no effect on electroshock threshold.

Linseed meal has long been known to be toxic when fed in large amounts. It was recently found by McGinnis & Polis (117) and by Kratzer (118, 119) that this toxicity could be removed by moistening linseed meal with water, incubating, and drying. It was subsequently reported by Kratzer & Williams (120) that the toxicity can be counteracted by the feeding of additional quantities of pyridoxine. The level of pyridoxine required by chicks on a diet containing 30 per cent of linseed meal is several times higher than

normal. It thus appears that linseed meal contains a pyridoxine antagonist which is destroyed by the process of soaking with water and drying.

An experiment to determine the effect of pyridoxine deficiency on human beings was carried out by Hawkins & Barsky (121), who used a simplified diet based on sucrose and "vitamin-free" casein and supplemented with the known vitamins except pyridoxine. A human subject was maintained on this diet for fifty-five days. During this time there was no disturbance of protein metabolism as measured by nitrogen balance. However, no measurements were made of xanthurenic acid and *N'*-methylnicotinamide excretion. A shift in the white blood cells towards lymphocytes was observed. This shift was restored towards normal when pyridoxine was added to the diet.

BIOTIN

The enzymatic role of biotin continues to be the subject of various investigations. In 1947, a number of laboratories independently demonstrated a function of biotin in the synthesis of aspartic acid (122, 123, 124). A role of biotin in the synthesis of oleic acid has also been indicated by Williams & Fieger (125), who described the ability of the latter substance to replace biotin in the nutrition of certain microorganisms.

The biotin requirement of *L. arabinosus* in the absence of aspartic acid was found by Potter & Elvehjem (126) to be approximately ten times as high as in its presence. In the presence of both oleate and aspartate no biotin was required. This indicated that the biotin requirement for aspartic acid synthesis was ten times as high as that for the synthesis of oleic acid. In the presence of a minimum amount of biotin which permitted synthesis of oleic acid, oxaloacetate could replace aspartic acid. This showed that transamination was not a limiting factor in the slow growth caused by biotin deficiency, and is interesting in view of the report by Lichstein & Christman (127) that in *Bacterium cadaveris* biotin functions in the reversible deamination of aspartic acid.

Interesting information has been obtained regarding the relationship between oleic acid and certain detergents. Williams & Fieger (128) found that a number of detergents were capable of replacing biotin and oleic acid. Williams *et al.* (129) have observed that certain detergents such as Tween 40 (a polyoxyethylene derivative of sorbitan esterified with palmitic acid) were inactive by

themselves for *L. bulgaricus* but greatly increased the growth response obtained with oleic acid. Tween 80, which is the corresponding ester of oleic acid, was capable of replacing oleic acid. Oleic acid alone exerted its growth promoting action through only a very narrow concentration range; at higher levels it became toxic. In the presence of the detergent Tween 40, oleic acid showed no toxic reactions.

While *L. arabinosus* required oleic acid only in the absence of biotin, other organisms such as *L. bulgaricus* and *L. leichmannii* were found by Williams *et al.* (129), and by Hutchings & Boggiano (130), to have an absolute requirement for oleic acid even in the presence of biotin.

Conflicting evidence has been presented regarding the role of biotin amino acid deaminases. Lichstein & Christman (127) reported that bacterial cells which had been exposed to pH 4 buffer at 20° to 30°C. showed a decreased ability to deaminate aspartic acid, serine, and threonine. This activity could be restored by the addition of either biotin or adenylic acid. The deamination of aspartic acid was reversible. Axelrod *et al.* (131) also found that cells of *E. coli* treated at pH 4 showed diminished deamination and decarboxylation of aspartic acid but the investigators did not obtain reactivation with biotin even with the same strain of *E. coli* as that employed by Lichstein (127). Hot water extracts of *E. coli* or of *L. arabinosus* could produce reactivation. Cell extracts of *L. arabinosus* which had been grown in presence of aspartic acid and were thus free from biotin were effective in producing reactivation. Thus it was possible to activate the aspartic acid-decarboxylation enzyme system with a preparation apparently free from biotin. It is difficult to reconcile the discrepancy between these two sets of results.

Further evidence for the role of biotin in decarboxylation was obtained by Ochoa *et al.* (132). They found that a biotin deficiency in the turkey produces a marked reduction of the enzyme which decarboxylates oxaloacetic acid to pyruvic acid and carbon dioxide. The concentration of a number of other representative enzymes was not decreased in biotin deficiency. No biotin could be found in the purified enzyme preparations. This observation is interesting in view of the finding of Axelrod (131) that biotin-free extracts of *L. arabinosus* cells could stimulate the production of carbon dioxide from aspartic acid by *E. coli* cells.

Biotin deficiency in ducks was found to reduce the rate of pyruvate and succinate metabolism by the heart ventricular tissue [Olson *et al.* (133)]. Injection of biotin in the deficient birds restored these values to normal, although addition of biotin to heart ventricle *in vitro* was without effect.

Earlier work by Trager (134) showed that the blood of various species contained a fat-soluble fraction (FSF) which was capable of replacing biotin in the nutrition of microorganisms. The fraction behaved like biotin in protecting chicks against the dermatitis of "egg-white injury" and in preventing the increased susceptibility to infection with malarial parasites which occurs during biotin deficiency. The fat-soluble nature of this compound, and its activity in the presence of excess avidin, indicate that it is not biotin. Fractionation of this factor by Axelrod *et al.* (135) showed that the biotin activity of FSF for human plasma could be explained on the basis of its content of oleic, linoleic, and arachidonic acids. Certain saturated fatty acids which were inactive by themselves had a synergistic action with these unsaturated fatty acids. While the biological activities of FSF and oleic acid appear to be qualitatively the same for microorganisms, there is a marked difference in their effect on chicks. Trager (136) found that FSF from hydrolyzed horse plasma reduced the severity of dermatitis in chicks produced by feeding egg white, while oleic acid was ineffective. Broquist & Snell (360) demonstrated that avidin could nullify the growth-promoting action of oleic acid for *L. arabinosus*.

The FSF of plasma was related by Trager (137) to avian malaria. Ducks which survived an infection with this parasite exhibited an increase in FSF during infection which reached its highest level during the period of decline in the number of parasites in the blood. In animals which died of infection, FSF fell to very low values just before death. When *Plasmodium lophurae* was cultured *in vitro* in suspensions of duck erythrocytes, wide ranges in biotin concentration had no effect on the rate of multiplication. Plasma protein fractions rich in FSF inhibited multiplication, while comparable fractions low in FSF did not.

DL-Oxybiotin was found by Krueger & Peterson (138) to be 50 per cent as effective as biotin for the nutrition of *L. pentosus* 124-2; oxybiotin was utilized as such and was not converted into biotin. These workers (139) compared the biological activities of "alpha biotin" prepared from egg yolk by Kögl with that of syn-

thetic DL-biotin, with five different microorganisms. After correcting for impurities in the "alpha biotin," and assuming that only D-biotin is active, the two samples exhibited the same biological activities for all five species employed.

The agar plate method was adopted to the quantitative assay of biotin using either *L. arabinosus* or *S. cerevisiae* by Genghof *et al.* (140) and by Williams (141). This method has the advantage of not requiring sterile samples and of producing a linear response to logarithmic increases in concentration over a 1,000-fold range.

Several publications have appeared on the role of biotin in reproduction. The addition of biotin to a purified casein diet supplemented with crystalline water soluble vitamins was found by Nelson & Evans (142) to improve "lactation performance" in the rat. It is interesting to note that a biotin deficiency was apparently produced in the adult rat solely by the stress of lactation. In the weanling rat, the use of avidin or a sulfonamide appears to be necessary for the production of symptoms of deficiency.

The biotin content of eggs from hens on an adequate diet was found by Couch *et al.* (143) to be about 600 $\mu\text{g.}$ per gm. of yolk and 100 $\mu\text{g.}$ per gm. of egg white. On a deficient diet these values rapidly decreased to 30 and 20 $\mu\text{g.}$, respectively, and when the biotin content of the yolk dropped to a level of 50 $\mu\text{g.}$ per gm., there was failure of embryonic development in eggs laid by hens in the same group. A comparison of biotin-deficient diets containing different carbohydrates showed that sucrose and lactose permitted a rapid drop in hatchability and in the biotin content of the egg. When dextrin was used, no symptoms of biotin deficiency occurred and the maintenance of a moderately high biotin content of the egg indicated that biotin synthesis was taking place, presumably in the intestinal tract. Following the administration of biotin to depleted hens, there was a rapid increase in hatchability of their eggs and in the biotin content thereof (144). Oleic acid was found to be inactive in increasing the hatchability of eggs laid by hens on diets deficient in biotin. The injection of aspartic acid into biotin-deficient eggs did not increase the rate of survival of embryos (145).

An investigation of biotin excretion before and after administration of this vitamin was used to study its clinical role by Oppel (146). After a test dose of 500 $\mu\text{g.}$, urinary excretion increased from an initial level of about 40 $\mu\text{g.}$ to a total of 250 to 350 $\mu\text{g.}$ per day. Simultaneous administration of 250 $\mu\text{g.}$ of avidin prevented an in-

crease in biotin excretion. A tolerance test on five patients with seborrhea showed four with a low rate of biotin excretion after a test dose. However, the administration of biotin was without beneficial effect on this skin disorder.

PANTOTHENIC ACID

Following the original observation by Lipmann *et al.* (147) that pantothenic acid is a component of coenzyme A, which functions in the acetylation of choline and sulfanilamide, a number of experiments have been made on the effect of pantothenic acid deficiency on the coenzyme A activity of animal tissue. Rats and ducks maintained on pantothenic acid-deficient diets were found by Olson & Kaplan (148) to show a reduction of 30 to 40 per cent in coenzyme A content of their tissues. Liver tissue from such deficient animals showed a decreased ability to utilize pyruvate. The lowered coenzyme A activity of the tissues was also reflected in the low ability of deficient rats to acetylate *p*-aminobenzoic acid as reported by Riggs & Hegsted (149, 150). Pantothenic acid in the form of coenzyme A was inactive microbiologically, but chicks were able to utilize coenzyme A as efficiently as pantothenic acid when both are given intraperitoneally, and 60 per cent as efficiently when given orally [Hegsted & Lipmann (151)]. This observation helps to explain the fact that microbiological assays for pantothenic acid have frequently yielded results much lower than those obtained by chick assay (152). It has been shown that pantothenic acid occurs in living cells primarily in the form of coenzyme A, and that this is not too completely hydrolyzed to microbiologically active forms by autolysis and by older methods of enzymatic hydrolysis.

The simultaneous action of purified alkaline intestinal phosphatase and a chicken liver enzyme was found by Neilands & Strong (153) to give values from 3 to 30 times higher than those obtained with the "mylase-P" enzymatic digestion method. However, it was reported that more complete liberation of pantothenic acid could be obtained by increasing the quantities of "mylase-P" used during digestion [Buskirk *et al.* (154)]. Lipmann *et al.* (147) found that pantothenic acid was involved in two types of linkages in coenzyme A; one of these was split by a phosphatase and the other by a liver enzyme. In view of this, it does not seem surprising that the single-step enzymatic digestion procedures used in the

past have failed to accomplish complete liberation of pantothenic acid from natural materials.

A conjugate of pantothenic acid was described which is more active for *Acetobacter suboxydans* than the free compound [King *et al.* (155)]. This conjugate, which occurs in heart muscle, is not dialyzable and does not have coenzyme A activity. In the most active preparations of these conjugates, which contained 5 per cent pantothenic acid, there were two to five molecules of glutamic acid per molecule of pantothenic acid [King & Cheldelin (156)].

Evidence has been obtained which indicates that pantothenic acid is concerned with the utilization of acetylmethylcarbinol by *Proteus morganii*. The compound is accumulated by this organism in the absence of pantothenic acid [McElroy & Dorfman (157)].

Pantothenyl alcohol, the alcohol analogue of pantothenic acid, has been found to be as efficient as pantothenic acid in increasing the urinary excretion of pantothenic acid activity as determined microbiologically in human subjects following administration of a test dose [Rubin *et al.* (158)]. When large doses of pantothenyl alcohol were given, the excretion of pantothenic acid was larger than after an equivalent amount of calcium pantothenate. This analogue had previously been shown to be effective in promoting growth and preventing achromotrichia in rats [Pfaltz (159)]. It is inactive either in promoting growth or in antagonizing pantothenic acid for *L. arabinosus* and *S. carlsbergensis* [Rubin *et al.* (158)] but is a weak antagonist for several other microorganisms [Shive & Snell (160)].

The pantothenic acid requirement for reproduction in the hen was found by Gillis *et al.* (161) to be between 7.5 to 10 mg. per kg. of diet. Egg production and maintenance of body weight were not adversely affected by levels of pantothenic acid as low as 1.5 mg. per kg. of diet. The requirement for growth of turkey poults was found to be approximately 10 mg. of pantothenic acid per kilo of diet by Kratzer & Williams (162), while that for the duck was reported to be 12 mg. per kg. of diet by Hegsted & Perry (60). The pantothenic acid requirement of the Shetland pony was estimated at 38 μ g. per kg. of body weight by Pearson & Schmidt (163).

A dietary source of pantothenic acid or riboflavin was reported by Olcese *et al.* (164) to be unnecessary for the rabbit. On diets low in either of these two vitamins, the fecal excretion was much greater than the dietary intake.

PTEROYLGLUTAMIC ACID

Research dealing with pteroylglutamic acid (PGA) was so productive during 1948 that an adequate treatment of the subject would occupy the space allotted for this entire review. In experimental animals, PGA deficiency was reported to produce such diverse changes as hydrocephalus, infarction of the spleen, impaired lactation, ulcerative colitis, inhibition of the growth of Rous sarcoma, and a failure to respond normally to estrogens. The apparent interchangeability of PGA and the liver principle in hemopoiesis in certain clinical anemias was studied but is not yet explained; the two factors appear to have separate roles in the nutrition of many species. The evidence for recognizing PGA as an essential human nutrient was presented (165). New compounds were synthesized which proved to be potent antagonists of PGA and some of these were used experimentally in the treatment of certain leukemias.

The literature on PGA and related compounds was reviewed (166).

Chemistry.—A series of articles by the Lederle and Calco groups, amplifying previous publications, described the isolation of PGA and pteroyltriglutamic acid ("fermentation *L. casei* factor"), their degradation, and the synthesis of pteric acid and pteroylglutamic acid (167 to 175). Further reports (176, 177) described the synthesis of pteroyl- α -glutamylglutamic acid, pteroyl- γ -glutamylglutamic acid, and pteroyl- γ -glutamyl- γ -glutamylglutamic acid. Comparative microbiological assays indicated the identity of the last-named compound with "fermentation *L. casei* factor."

A synthesis of PGA by condensing 2-amino-4-hydroxy-6-hydroxymethylpteridine with *p*-aminobenzoylglutamic acid was reported by Karrer & Schwyzer (178). These authors also described the formation of PGA by the condensation of glyceraldehydetolulol sulfuric acid ester with 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoylglutamic acid in the presence of sodium iodide.

Clinical.—The clinical use of PGA during 1948 was marked by debate regarding its effect on the neurological changes in pernicious anemia (179). It was suggested without experimental evidence that PGA may function as a metabolic antagonist for glutamic acid and may "competitively interfere with the nutrition of the spinal cord" (180). However, it was shown by Franklin *et al.* (181) that PGA was not a glutamic acid antagonist as indicated by

growth of *L. casei* or *S. faecalis* R or by respiratory measurements with brain or kidney slices. In the respiratory experiments, PGA had no effect when used at a level of more than 100 times the highest concentration reported to have been found in human blood. Grossowicz (182) found that PGA, glutamic acid, or glutathione were interchangeable in reversing an inhibition of *Staphylococcus aureus* caused by glutamine; subsequent work by Hughes (323) indicated that the inhibition was due to zinc in the glutamine preparation.

"Refractory megaloblastic anemia," resistant to potent liver extracts, was described by Davidson (183), who drew attention to the good responses which were produced by "proteolyzed liver" or by PGA. Many of the cases were associated with pregnancy, child-bearing, or sprue. At present, there is insufficient evidence to determine whether or not the effective factor in "proteolyzed liver" is PGA. Spies and co-workers (184) studied 32 cases of "nutritional macrocytic anemia," in which free hydrochloric acid was present in the gastric juice after histamine stimulation, thus contrasting the syndrome with pernicious anemia. Satisfactory remission and maintenance were obtained with PGA, and no neurological symptoms were seen during the observation period of 18 to 24 months. Goodall and co-workers (185) used PGA, which they stated was superior to liver extract, in the treatment of "nutritional macrocytic anemia"; several of the cases occurred in pregnancy. Davidson & Girdwood (186) noted the development of signs which were attributed to a deficiency of the vitamin B complex in patients with pernicious anemia and sprue treated with PGA alone. The recommended the use of both PGA and liver in the treatment of sprue.

Neurological disturbances were noted in eight out of ten cases of pernicious anemia which were treated for various periods ranging up to 12 months with PGA alone (187). While as a general rule, PGA does not protect patients with pernicious anemia against such disturbances, occasionally cases are recorded in which neurological improvement was noted during treatment with PGA alone (188).

The conclusion may be drawn that the neurological changes in pernicious anemia are usually due to a lack of a factor present in concentrated liver extracts; patients treated with PGA alone are brought into hemopoietic remission which may tend in turn to

bring the nervous symptoms and the glossitis into greater prominence (189). Concurrent administration of PGA and liver extract was reported to produce higher red cell counts than were obtained with liver extract alone, and no evidence of neurologic relapse occurred (179).

In other clinical studies, PGA was reported to relieve certain blood dyscrasias, including a case of "achrestic anemia" (190). Metabolic and respiratory studies (191) with pernicious anemia patients indicated that PGA increased their nitrogen retention. No such effect was produced consistently in normal controls.

Animal nutrition.—The occurrence of hydrocephalus in about 2 per cent of the young offspring of rats on a purified PGA-deficient diet was noted by Richardson & Hogan (192). The abnormality was found by O'Dell and co-workers (193) to be largely prevented by the addition of PGA to the maternal diet. A high incidence of infarction of the spleen in young rats fed a PGA-deficient diet was noted by Asenjo (194). No lesions were noted in control rats which had received 5 μ g. of PGA daily. Deficiencies in a corn-soybean meal-5 per cent alfalfa meal diet for rats were studied by Maruyama & Phillips (195), who found that additions of PGA, lysine, and methionine improved reproduction and lactation on such a diet.

The effects of PGA and pyridoxine on radiation sickness were studied by Goldfeder *et al.* (196), who found that 15 μ g. of PGA or 50 μ g. of pyridoxine injected daily from one week before until two weeks after x-radiation extended significantly the life spans of the mice as compared with unsupplemented controls. No such protection was afforded by PGA to irradiated swine in studies by Cronkite *et al.* (197), who found that the average survival time of supplemented or unsupplemented animals was between 14 and 15 days.

The interchangeability of PGA and liver extract or additional casein in relieving certain blood dyscrasias in rats on a low-casein diet, first reported by Daft (198), was confirmed for casein by Shehata & Johnson (199) and for liver extract by Mushett & Emerson (200). The latter workers also noted that either PGA or purified antipernicious anemia liver extracts would relieve the leukopenia and granulocytopenia produced in rats by feeding purified diets which contained 1 per cent of sulfathiazole. The beneficial effect of casein, PGA, or liver extract on low-protein

anemia in rats was also noted by Aschkenazy & Aschkenazy-Lelu (201). Colitis in monkeys in both the acute and chronic stages of PGA deficiency was described by Rinehart & Greenberg (202).

Continuing their nutritional studies on reproduction in rats, Nelson & Evans (203, 204) demonstrated the occurrence of a severe leucopenia and granulocytopenia, often accompanied by anemia, in lactating rats which received a purified diet containing succinylsulfathiazole. The blood dyscrasia and loss in weight were prevented by administering PGA. Feeding a liver fraction resulted in additional improvement. Simultaneous investigations by Sica and co-workers (205) indicated a beneficial effect of PGA on reproduction and lactation in rats while xanthopterin was reported to improve lactation.

Pigs were fed a basal purified diet containing 2 per cent of succinylsulfathiazole (206), under which conditions they developed a normocytic anemia which was prevented by PGA. However, Johnson *et al.* (207) could not consistently produce a clear-cut PGA deficiency in young pigs on purified diets even when two per cent of succinylsulfaphthalidine was added, although in one experiment a beginning PGA deficiency was indicated by a lower growth rate and lighter hair coats.

Chicks or turkey poults were found to have low reserves of PGA when they were hatched from eggs laid by breeding hens which were fed diets low in PGA (208, 209). A marked increase was noted in the antifibrinolysin activity of the blood of chicks which received a diet deficient in PGA (210) as compared with control chicks. Norris & Majnarich (211) reported that xanthopterin was more effective than PGA in causing an increase in the rate of red and white cell proliferation in bone marrow cultures. Xanthopterin when injected relieved the anemia which occurred in rats fed a purified diet containing 1 per cent of sulfathiazole. Studies with narrow tissue *in vitro* showed that certain other pterin compounds had some activity and that xanthopterin-7-carboxylic acid and 2-amino-4-hydroxy-7-methylpteridine were inhibitory, the latter reversibly so. In contrast, Pritchard (212) found that rats on a purified diet plus 1 per cent of sulfathiazole developed a hemolytic anemia which was not reversed by xanthopterin or PGA. On a diet containing 1 per cent of succinylsulfathiazole, rats which had been made anemic by repeated hemorrhage responded to PGA but not

xanthopterin. The anemia produced in rats on a "chow" diet by infection with *Bartonella muris* was found to be diminished in severity when PGA, 50 μ g., was injected daily (213). The administration of streptomycin to rats and chicks produced granulocytopenia and macrocytic anemia with hypoplasia of the bone marrow. The changes were reversed by administering PGA (214).

Experiments with young dogs on a purified diet containing 1 per cent succinylsulfathiazole indicated that nicotinic acid, PGA, and a factor present in concentrated liver extracts were all necessary for hemopoiesis and "general health." On high levels of protein, the need for PGA could not be shown (215).

Attempts failed to confirm the report of Davis (216) that PGA and liver extract increased plasma cholinesterase in dogs. Negative findings were reported by Kunkel and co-workers (217), who also found that acetylcholine and physostigmine did not induce anemia-like changes in dogs. Hawkins (218) found no increase in cholinesterase activity when PGA was incubated with plasma or was

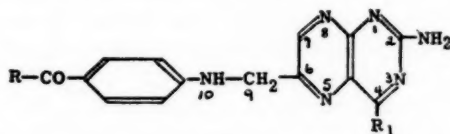


FIG. 2.—Pteroylglutamic acid; R = glutamic acid radicle, $R_1 = OH$.

administered to dogs. The mealworm, *Tenebrio molitor*, was found to need PGA or PGA conjugate for growth and survival and also an unidentified factor designated "B_T" (219).

Only small amounts of PGA activity were found in the urine and feces of monkeys on a PGA-deficient diet (220). The amounts were not significantly increased by feeding 0.1 mg. of either pterioic acid, PGA, or PGA as conjugate and even when 1 mg. of PGA was fed, the urinary excretion was only about 1 per cent of the oral dose and the fecal excretion was much less.

Antagonists.—A number of synthetic substances have been found to show varying degrees of biological antagonism against PGA. The numbering system used (221) in describing analogues of PGA is shown in Fig. 2.

Space does not permit of a detailed discussion of all the individual PGA antagonists. A list of the types is as follows:

Class	Type	Example	Reference
1	Purine analogues	2-aminopurine	(222)
2	Pteridine compounds	2,4-diamino-6,7-diphenyl- pteridine	(223, 224, 225)
3	PGA with difference substituents on pteridine nucleus or on side chain or on both	4-amino-PGA N ¹⁰ -methyl-PGA 4-amino-N ¹⁰ -methyl-PGA	(226) (227) (228)
4	Pteroylamino acids other than PGA	Pteroylaspartic acid	(229, 230)
5	PGA with modifications of pteridine nucleus	Quinoxaline-2-carboxyl p-aminobenzoylglutamic acid	(231)
6	PGA analogues with carboxyl of PAB replaced by sulfonyl	N-[4-((2-benzimidazol)-methyl-amino)-sulfonyl]-glutamic acid	(232)

Information regarding the anti-PGA action of these various compounds relates only to their effect in the microbiological assay procedure in the cases of the purine analogues (222) and the sulfonyl compound of Edwards *et al.* (232). Among the various pteridines studied by Daniel *et al.* (225), 2-amino-4-hydroxy-6,7-dimethylpteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine appeared to have a slightly anti-PGA effect for chicks at comparatively high levels when added to the diet. The compound 2-amino-4,7-dihydroxypteridine-6-carboxyl-p-aminobenzoylglutamic acid was toxic to rats in a single dose of 10 mg. (231). The animals were protected by 10 µg. of PGA. The antagonist pteroylaspartic acid was ineffective in producing blood regeneration in two cases of sprue (233).

It was reported by Hertz (234) and by Franklin *et al.* (235) that crude "x-methyl" PGA (236), when fed to chicks, blocked the response of the oviduct to diethylstilbestrol. The block was removed by administering a suitable quantity of PGA (234). Monkeys on a PGA-deficient diet were found to show little or no response to the injection of estradiol benzoate at a level which produced highly characteristic external changes in control animals (237). Several PGA antagonists were reported to inhibit the action of "dopa" decarboxylase (238).

Crude "x-methyl" PGA was used by Cartwright and co-workers (239) to produce PGA deficiency in pigs on a purified diet. The casein in the diet was demonstrated to contain the "extrinsic factor." The animals developed a severe anemia accompanied by

bone-marrow changes. The anemia did not show a marked or consistent response to liver extract, but was quickly relieved by small doses of PGA. Heinle and co-workers (240) used the same PGA antagonists but noted that the diet in their experiments was "essentially free of extrinsic factor." Three pigs in this study were treated with PGA alternated with either liver extract or crude casein and the results were suggestive of a double deficiency of PGA and the antipernicious anemia factor. Weir and co-workers fed the same antagonist to mice (241) which developed a dyscrasia marked by a reduction of all cellular elements of the blood and an arrest of maturation in the bone marrow. Treatment with PGA restored the conditions to normal.

The crude "x-methyl" PGA was found by Goldsmith and co-workers (242) to prevent the metamorphosis of *Drosophila melanogaster* larvae. The effect was reversed by PGA. A similar but irreversible prevention of metamorphosis was obtained with 4-amino-PGA; this compound decreased the response of the oviducts of female frogs to estradiol to which the response in other frogs was actually increased by pretreatment with PGA (243).

The most potent of the PGA antagonists yet described is 4-amino-pteroylglutamic acid (4-amino-PGA) (226), $R_1 = NH_2$, which was reported to cause the death of mice within a few days when fed at levels of 1 mg. or more per kilo of diet (244), even when levels of PGA up to 100 mg. were added per kilo of diet. Some reversal was obtained with PGA when the level of the antagonist was 0.3 mg. Minnich & Moore (245) found that 4-amino-PGA in small doses produced a profound anemia and leucopenia, terminating fatally, in guinea pigs. Liver extract had no effect on the action of the antagonist, and PGA did not prevent the anemia. Swendseid *et al.* (246) studied the effect of various PGA analogues on rats. The most effective compound in producing anemia and leucopenia was 4-amino-PGA, and 2,4-diamino-6,7-diphenylpteridine was also active. Reversal of the former compound by PGA was mentioned. Oleson and co-workers (247) also found that 4-amino-PGA was toxic for rats, and in addition reported studies with chicks in which reversal of the effects of 4 mg. of 4-amino-PGA per kilo of diet was obtained with 96 mg. of PGA. Children with acute leukemia were treated with 4-amino-PGA, 1 mg. daily, by Farber and co-workers (248). Of 16 cases, 10 showed improvement during three months. Toxic reactions to the drug were described in this

report and in other accounts by Jacobson *et al.* (249), Pierce & Alt (250), Berman *et al.* (251), and Taylor *et al.* (252). The changes included stomatitis, dermatitis, alopecia, pharyngitis, ulceration of the gastro-intestinal tract and of the buccal, vaginal, and rectal mucosa. The substance had a markedly depressant effect on the bone marrow.

A dietary deficiency of PGA was found to inhibit the growth of Rous sarcoma in chicks (253). Similar results were observed with chicks on a commercial diet to which was added either of the PGA antagonists 4-amino-pteroylaspartic acid and 4-amino-pteroyl-D(-)-glutamic acid (254).

Cytological changes caused by administering PGA antagonists to the developing chick embryo were described by Wagley & Morgan (255). They found that 5 μ g. of 4-amino-PGA or 10 μ g. of 4-amino-N¹⁰-methyl-PGA produced the changes, and that the action of these compounds was prevented by the prior administration of 12.5 mg. of PGA. "Vitamin B₁₂," 5 μ g., or liver extract had no preventive effect.

Blood-cell cultures grown in the presence of "x-methyl" PGA or 9-methyl-PGA showed marked erythrophagocytosis which was not observed when PGA was also added (256).

PGA conjugase.—The presence of a "conjugase inhibitor" in the diet did not decrease the efficiency of utilization of PGA conjugate for leucocyte maturation in rats (257). However, the presence of the inhibitor appeared to diminish somewhat the rate of urinary excretion of PGA following the administration of the conjugate.

The PGA conjugase in the blood of chickens and turkeys was observed by Schweigert (258) to have a pH optimum in the neighborhood of 6.0 to 7.0. Buyze & Engel (259) reported that PGA conjugate was changed by incubation with normal human gastric juice at pH 2, but no PGA was liberated. Normal duodenal juice had no conjugase activity. The activity of chicken pancreas conjugase was found by Stokstad and co-workers to be inhibited by *p*-aminobenzoyltriglutamic acid (260). Studies by Kazenko & Laskowski with concentrated preparations of chicken pancreas conjugase indicated that the enzyme should be classified as γ -glutamic acid carboxylpeptidase, requiring at least two terminal glutamic acid molecules in the peptide chain (261). The existence in rat liver of a precursor of "folic acid," as measured by assay with

S. faecalis R, other than PGA conjugate was reported by Olson and co-workers (262). They found that "folic acid" activity of rat liver homogenates was increased by incubating at pH 7, but was not increased at pH 4.5 by autolysis or by digesting with hog kidney conjugase. This confirmed previous work by Wright and co-workers which had shown that when rat liver was incubated alone at various pH values, a maximum amount of "folic acid" activity was liberated at pH 7 (263). Studies by Hall indicated that mouse skin or cat liver liberated small quantities of a folic acid-like factor when incubated with histidine at pH 7.4 (264).

Assay.—The results of a collaborative study of the microbiological assay of PGA were reported by Flynn (265) in an article which contains discussions of many details of the method. Olson and co-workers presented results which indicated that a hog kidney conjugase liberated more PGA activity from natural materials than did taka-diastase (266). The presence of PGA conjugate in taka-diastase from which PGA may be liberated by conjugases present in food or tissue samples has received comment by Simpson & Schweigert (267). Hodson (268) noted an inhibitory action of various proteins on hog kidney conjugase.

Miscellaneous.—Xanthopterin oxidase and xanthine oxidase were found to be inhibited by PGA in studies by Kalckar & Klenow (269), who also noted that the inhibitory effect of PGA was removed by a preliminary treatment with "milk xanthopterin oxidase." Subsequent experiments (270) showed that the inhibitory power of PGA was greatly reduced by purification and that 2-amino-4-hydroxy-6-formylpteridine, a photofission product of PGA, was a highly potent inhibitor of the two oxidases and also of quinine oxidase. Milk and rat liver contained an enzyme which converted the pteridine into a noninhibitory compound. The xanthine oxidase content of liver tissue was found to be higher than normal in PGA deficient chicks (358).

It was noted by Hitchings *et al.* (271) that bromouracil could reverse the inhibitory action of nitrouracil against PGA in studies with *L. casei*. This observation was used to illustrate the point that substances which reverse an inhibition of bacterial growth are not necessarily products or substrates of normal metabolism. On the basis of its greater activity than PGA in reversing the inhibitory effect of an antagonist, N¹⁰-formyl-PGA was postulated to be a "functional derivative" of PGA (272). The further suggestion

was made that N¹⁰-formyl-PGA might be superior to PGA in the treatment of pernicious anemia. However, Spies and co-workers (233) showed independently that N¹⁰-formyl PGA was much less effective than PGA in the treatment of pernicious anemia or nutritional macrocytic anemia.

The conversion of PGA into pteric acid by degradation with an organism which appeared to be similar to *Flavobacterium buccalis* was described by Lemon *et al.* (273). The inhibitory action of sulfadiazine on the psittacosis organism was found to be antagonized competitively in the yolk sac by 6-aminobenzoic acid and non-competitively by PGA (274).

When *L. casei* was grown on a medium partially deficient in PGA, the bacterial cellular material was found to be lower than normal in its content of desoxyribonucleic acid but not in its content of ribonucleic acid. Comparable deficiencies of riboflavin or biotin had no such effect (359).

ANTIPERNICIOUS ANEMIA FACTOR, "B₁₂," ANIMAL PROTEIN
FACTOR, COW MANURE FACTOR, "FACTOR X,"
AND ZOOPHERIN

Vainly sought for many years, the antipernicious-anemia factor (APA) of liver extract was finally separated during 1948 in the form of red crystals (275, 276, 277). The name "vitamin B₁₂" was suggested (275). The pathways of various investigations of the nutrition of rats and chickens converged upon the factor. It was found to promote the growth of chicks (278) and rats (279) which were fed diets low in the "animal protein factor." In another approach, concentrates of microbial origin, standardized against liver extract by "animal protein factor" assay with chicks, were found to produce hematologic remissions in patients with pernicious anemia (280).

Isolation and properties.—An undescribed microbiological assay method employing *L. lactis* Dorner (281) was used by Rickes and co-workers in their fractionation studies. Smith (276, 277) appears to have accomplished the feat of isolating APA by the use of tests with human patients, coupled with the observation that active fractions were characterized by a pink color which could be followed by chromatography. Earlier work had demonstrated that amorphous preparations could be obtained from liver extract which were active in the treatment of patients with pernicious

anemia at a daily dosage rate of 0.3 mg. (282) or a total dosage of 1 mg. (283), therefore, it was anticipated that the pure factor would be effective in extremely small amounts. Such proved to be the case; a single injection of 3 μ g. of "B₁₂" was found by West (284) to produce a hemopoietic response in a case of pernicious anemia. The crystalline compound blackened without melting when heated to 300°C. (275). The presence of 4.0 per cent of cobalt was found by Smith (277), who noted that if each molecule of the compound contained one atom of cobalt, the molecular weight would be about 1,600, which was in good agreement with the value obtained by x-ray crystallographic procedures. His analyses indicated the presence of three atoms of phosphorus. Rickes and co-workers (285) also noted the presence of cobalt, phosphorus, and nitrogen and the absence of sulfur in "B₁₂." "Half-maximal growth" of *L. lactis* was supported by 0.013 m μ g. per ml. of culture medium. The microbiological activity of the factor was not changed by autoclaving for 15 min. at 121°C., but it was slowly inactivated by standing in dilute sodium hydroxide or hydrochloric acid at room temperatures.

It was reported (286) that the infrared absorption spectrum of the crystalline APA facta was measured in England and that the presence of PO, NH, and OH groups was thus indicated together with the absence of aliphatic CH and the probable presence of aromatic CH.

The existence of more than one molecular species having similar biological effects is indicated by the chromatographic separation of two pink bands with biological activity (276) and by the non-dialyzability of the factor in cow manure (287) as contrasted with the dialyzability of a similarly-assayed factor prepared from liver (288).

Clinical.—During the past 20 years, much information has accumulated regarding the clinical effects of antipernicious-anemia liver extracts upon the megaloblastic anemias. It was not to be expected that the use of the isolated APA factor would produce results beyond those which had previously been obtained in patients by administering liver extract. It was of great interest to determine whether "vitamin B₁₂" would alleviate or arrest the progress of the neurological changes which are frequently encountered in pernicious anemia. Smith (276) reported that incompletely purified material was effective against the spinal cord symptoms.

West (284) announced that three patients showed rises in reticulocytes, red cell count, and hemoglobin following the administration of 3, 6, and 150 μ g. of "vitamin B₁₂" respectively. In studies with impure preparations standardized with *L. lactis* Dorner it was found that single doses corresponding to about 2 to 4 μ g. gave strong hematological responses. Results of a confirmatory nature were reported by Spies and co-workers (289, 290), who also found preliminary indications that two patients with "nutritional macrocytic anemia" and three with sprue showed responses to the factor. Stone & Spies (291) reported that the mucous membrane lesions in two patients with pernicious anemia were promptly relieved by either liver extract or "vitamin B₁₂." The lesions did not respond to thymidine or folic acid and were thought to be associated with the neurological changes in combined system disease. Berk and co-workers (292) described a patient with combined system disease who showed a hematological and neurological response to the daily administration of 5 μ g. of "vitamin B₁₂" daily for eight days. There was a marked improvement during this period, however, when the injections were discontinued, the patient showed neurological regression which started within the remarkably short time of seven days. Further treatment led to the resumption of the improvement. The patient was sensitive to liver, but showed no adverse reaction to "B₁₂." Three patients (293) showing combined system disease received single intramuscular injections of 15 μ g. of "B₁₂"; one of them showed a marked improvement in neurological symptoms and the other showed less pronounced changes. A fourth patient gave a marked response to liver extract. Hall & Campbell (294) and Bethell and co-workers (295) also have presented evidence that patients with combined system disease showed an over-all response to "vitamin B₁₂" although an exception was noted (294). A case of puerperal macrocytic anemia grew worse during treatment with "vitamin B₁₂," but responded promptly to the administration of pteroylglutamic acid (295). The response of a pernicious anemia patient to "vitamin B₁₂" was apparently "blocked" by administering 4-amino-PGA, which is an antagonist for PGA. This interesting observation may suggest that "B₁₂" is hemopoietically ineffective in the absence of PGA.

Berk and co-workers (296) found that the hemopoietic activity of orally administered "B₁₂," 5 μ g. daily, was increased by simultaneously giving 125 to 150 cc. of normal human gastric juice. This suggested that the so-called "extrinsic factor" might be identi-

cal with or closely related to "B₁₂" and relegated the function of the "intrinsic factor" to facilitating the uptake of "B₁₂" or related compounds from the gut.

In addition to *L. lactis* Dorner, other microorganisms which have been used for the assay of APA include *L. leichmannii*, 313, ATCC 7830 (297), and *Euglena gracilis* (298), while another strain of *L. leichmannii*, ATCC 4797 (299) was observed to respond to concentrated preparations of the "animal protein factor." Daniel and co-workers (300) found that liver extract was active for *L. casei*, but they stated that it would seem impossible that this "animal protein factor activity" is "identical with the factor required in the treatment of pernicious anemia." A relation between thymidine and APA was indicated by Shive and co-workers (301) and Wright *et al.* (302), who found that thymidine would replace liver extract in promoting the growth of *L. lactis* Dorner. For half-maximum growth, about 0.5 μ g. of thymidine per cc. of medium was required by three lactic acid organisms (303). Using *L. leichmannii* 313, either 0.25 or 0.5 μ g. of thymidine or 0.075 μ g. of APA was needed per cc. of culture medium for half-maximum growth (297), perhaps suggesting a catalytic function for APA in the formation of thymidine by certain lactobacilli as proposed elsewhere (301, 302). By contrast, thymidine did not promote the growth of *Euglena gracilis* which needed no B-complex factors added to the medium in addition to thiamine and APA (298). "Half-maximum response" of this organism was produced by 0.01 μ g. per ml. of culture medium.

Various investigations in poultry nutrition provided evidence during 1948 which confirmed and extended previous findings relative to the presence of the "animal protein factor" in fish meal (304, 305), "fish solubles" (306 to 309), hen manure (310, 311), and cow manure (287). The presence of this factor was reflected by such criteria as improved growth and higher survival rate of chicks, and increased hatchability of hens' eggs. The presence of the factor in a sample of soil was noted (312). Indications of a multiple nature for the factor were noted by Hill and co-workers (313); dried whey produced a partial response in chicks, but fish solubles gave a more complete response. An elevation of the nonprotein-nitrogen in the blood of deficient chicks when "vitamin-free-casein" or a soybean protein fraction were added to the basal diet was noted by McGinnis and co-workers (314). This elevation was prevented by feeding a liver fraction.

Rats on a diet containing yeast and wheat germ as B vitamin sources were found some years ago by Mapson (315) to develop a deficiency which responded to liver. Subsequent work resulted in similar findings in various laboratories, and it was briefly reported by Cary, Hartman and their co-workers that very small doses of antipernicious anemia liver extract were effective in promoting the growth of rats on a purified diet containing alcohol-extracted casein and yeast (316). The deficiency signs were accentuated by adding soybean meal or more casein to the diet and were attributed to the lack of an unidentified "Factor X." Zucker & Zucker (317) used various high-protein diets for rats and noted that the young born of females on such diets showed a high incidence of mortality soon after weaning. The syndrome was marked by slow growth, a high incidence of mortality, high blood urea, a low white cell count, kidney hypertrophy, and other visceral changes and was prevented or cured by a liver fraction, "fish solubles," crude casein, or a concentrate of the "cow manure factor" (318). The name "Zoopherin" was proposed for the missing dietary factor on the basis of the conception that it was associated exclusively with animal materials (317, 319, 320). However, it seems that the factor is produced by various bacteria (280, 321, 322) and that the sole claim to "Zoopherin" on behalf of the animal kingdom cannot be sustained.

CHOLINE

The presence of an enzyme in cabbage leaves which appears to split choline from its phosphoric acid linkage in phospholipids was noted by Hanahan & Chaikoff (324). The choline acetylase system was re-examined by Nachmansohn & Weiss; acetate rather than citrate was found to be used in the synthesis (325).

A deficiency of lipotropic factors in the diet was considered to be a predisposing factor to cirrhosis among patients studied by Fernando *et al.* (326).

Studies by Zilversmit *et al.* (327) showed that choline increased the turnover rate of lecithin in the liver but not that of lecithin or sphingomyelin in the plasma. This indicated that choline acted by stimulating the utilization of fats within the liver itself rather than by increasing fat transport via phospholipids. The administration of either ethanolamine, methylethanolamine, dimethylethanolamine, or choline was found by Artom & Cornatzner to increase the rate of formation of total phospholipids in rats (328).

A choline deficiency in mature rats was shown by Peet & Sampson (329) to cause a loss of contractility of the uterus, and partial atrophy of the uterus and ovaries. An investigation by Hardwick & Winzler (330) of diethylcholine and ethionine indicated that these two compounds were lipotropic and prevented hemorrhagic kidney degeneration, but did not support growth. Ethionine was toxic to rats but no toxicity with diethylcholine was observed. Morrison & Rossi (331) noted that the atherosclerotic lesions in the aortas of cholesterol-fed rabbits disappeared following the feeding of choline.

Studies by Johnson & James (332) indicated that baby pigs required choline even though the purified diet contained 30 per cent casein. The deficiency symptoms observed included fat deposition in the liver, and a reduction in red blood cell count.

Investigations with dimethylthetin and dimethyl- β -propiothetin showed that these two compounds were highly active as a source of labile methyl in the formation of methionine from homocysteine (333) and were effective as lipotropic and kidney antihemorrhagic factors (334). Dimethyl- β -propiothetin was also effective in promoting growth in rats on diets which were low in labile methyl and contained homocystine (335).

MISCELLANEOUS

Rutin.—Clinical studies with this flavonol glucoside showed that it does not produce any marked improvement in the retinal hemorrhages of diabetes [Levitt *et al.* (336)]. Rodriquez & Root (337) in another study reported that while capillary fragility in diabetic patients could be brought to normal by prolonged treatment with rutin, there was no definite effect on the retinitis. In the guinea pig, Ambrose (338) noted that quercitin or rutin exhibited a sparing action on subminimal amounts of ascorbic acid.

In view of the previously advanced view that "vitamin P" acts *in vivo* by prolonging the effect of epinephrine, a number of experiments have been made to test this hypothesis. A study of a number of related compounds by Clark & Geissman (339) showed that many compounds were more active than rutin or "citrin" and that activity appeared to be related to reducing power and to the metal complexing or chelating capacity.

p-Aminobenzoic acid.—The growth of a certain mutant strain of *Neurospora* was shown to be stimulated by sulfanilamide.

Zalokar (340) crossed this strain with a "*p*-aminobenzoic acid-less" strain to obtain the double mutant. This strain required *p*-aminobenzoic acid in low concentrations but was inhibited by higher concentrations. On the basis of this and other data, he concluded that sulfonamide was not an essential factor but promoted growth by inhibiting some of the toxic reactions produced by the large amounts of *p*-aminobenzoic acid which were produced by the organism.

"*Vitamin B₁₃*".—A concentrate was obtained from dried distiller's solubles and from rice polishings concentrate which was active in stimulating growth of rats at levels of 10 μ g. per day. This factor which was said to be distinct from all other known factors was designated "*vitamin B₁₃*" by Novak & Hauge (341, 342). The purified basal diet containing exhaustively-extracted casein which they employed would be expected to be deficient in "*vitamin B₁₂*." However, the reported solubility of "*vitamin B₁₃*" in ether and the absence of a red color sharply differentiated it from "*vitamin B₁₂*."

The "monkey antianemia factor" was reported by Ruegamer *et al.* (343) to be distinct from PGA and the antipernicious anemia factor. Experiments with foxes and mink by Schaefer *et al.* (344, 345, 346) showed that these animals require an unknown factor present in fresh liver and fresh milk. Deficiency symptoms include suboptimal hemoglobin levels, loss in body weight, dull matted fur, and, in the case of the mink, paralysis of the hindquarters. On the basis of its distribution in foods, chemical stability, and the character of the deficiency symptoms, this factor was said to resemble the antianemic factor for monkeys reported by Cooperman *et al.* (347). However, the possibility of an insufficient level of PGA in the basal diets appears to exist.

Unknown factors.—Colio & Baff (348) found that *S. lactis* R required an unknown factor present in malt sprouts, and distinct from pteroylglutamic acid and streptogenin. A "methionineless" strain of *Neurospora crassa* was found by Graham *et al.* (349) to respond to an unidentified factor in certain protein hydrolysates. Growth in rabbits was reported by Kunkel *et al.* (350) to be improved by adding 4 per cent of a crude liver concentrate to a purified diet; PGA, biotin, *p*-aminobenzoic acid, and cystine were ineffective.

A factor reported by Sauberlich & Baumann (351) required for

the growth of *Leuconostoc citrovorum* 8081 was present in widely varying amounts in antipernicious-anemia liver extracts, but appeared to differ from the animal protein factor required by chicks.

Strepogenin.—The strepogenin activity of L-serylglycyl-L-glutamic acid, previously described by Woolley (352), was confirmed by Krehl & Fruton (353). An indirect approach to the chemistry of strepogenin was made by Woolley (354) in a study of the peptides in insulin. Since strepogenin was found to contain amino groups which are free in the original protein, the separation of derivatives of strepogenin was attempted by preparing the dinitrophenyl derivatives of insulin and liberating the peptides by proteolytic digestion. The yellow peptides obtained by the procedure were fractionated by counter-current liquid-liquid extraction and several crystalline peptides were obtained. Since strepogenin activity seemed to be associated with glutamic acid in peptide form, it was hoped that a study of the glutamic acid-containing peptides from insulin might shed some light on the structure of strepogenin. The peptides obtained by this procedure were all biologically inactive because of the dinitrophenyl group. This method is of interest not only by reason of its relation to the strepogenin problem but because it offers a promising approach to the study of peptide structure.

Stress factors.—Ershoff and co-workers have shown that factors of stress, such as the feeding of thyroid, exposure to cold, and the administration of atabrine, increase the nutritional requirements of the rat. Thyroxine, thyroglobulin, and iodinated casein were shown by Ershoff and McWilliams (355) to have the same effect as desiccated thyroid in depressing the growth of rats. Growth was restored by the addition of liver residue. It has not been established whether the unknown factor in liver residue is identical with "vitamin B₁₂." Smith *et al.* (356) reported that whole liver powder produced a slightly greater growth response in rats raised in the cold than those grown under normal temperatures. The feeding of atabrine produced a marked depression in growth [Ershoff (357)]. Addition of the synthetic B complex vitamins produced a partial restoration of growth while whole liver powder effected a larger response. Yeast was inactive. The addition of the same supplements to the diet without atabrine produced only very small increases in weight.

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NUTRITION

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A regrettable mishap prevented the appearance of a review on Nutrition in Volume XVII (1948). The present review, therefore, should attempt to cover the two years beginning in the Fall of 1946, as well as some war-time material only lately available. Because of the general expansion in nutritional research and the reporting of an enormous mass of war and postwar data the output for this period greatly exceeds that of any previous two-year period. This review is restricted to problems of immediate concern in human nutrition with emphasis on semistarvation. Even within this limitation, it has been necessary to omit topics such as infant feeding, specific nutrients in the treatment of disease, intermediary metabolism, and the chemistry of foods and individual nutrients. Historical and sociological discussions are interesting (e.g., 60, 318) but are inappropriate for discussion here. The contributions cited here have been chosen because, in the mass, they provide a reliable guide to new facts and new approaches in human nutrition.

The 1947 review by McHenry & Leeson (254) expressed a critical and realistic attitude which is increasingly evident in recent discussions on nutrition (79, 204, 353). Excesses of enthusiasm over advances in nutritional knowledge have been tempered by the results of controlled studies on man and, especially, by the sobering experiences of practical nutrition during and following the War. There is an increasing reluctance to extrapolate to human nutrition from findings on laboratory animals. Nutritional status is more frequently investigated directly rather than inferred from dietary estimates and "standards" (257, 362). The importance of calories is being rediscovered and the role of adaptation is beginning to be appreciated. It may be hoped and expected that these trends will continue.

SOURCES OF LITERATURE

Nutrition Abstracts and Reviews continues to be the most important single compilation of literature; taken together with *Chemical Abstracts* and the *Quarterly Cumulative Index Medicus*

(when it recovers from its recent default), few significant papers should be missed. The newer abstracting journals, *Abstracts of World Medicine* and *Excerpta Medica*, as yet provide small coverage on nutrition. The reference system offered by *Voeding* is valuable for those who read Dutch. Among new journals are *Annales de la Nutrition et de l'Alimentation* (Paris) (13), the *Chinese Journal of Nutrition* (Shanghai), *Arquivos Brasileiros de Nutrição*, *Revista Nutricion* (Lima, Peru) and the *British Journal of Nutrition*; this last-mentioned seems particularly promising. There should shortly be available a *Handbook of Clinical Nutrition* published under the auspices of the National Research Council, and a new edition of the American Medical Association *Handbook of Nutrition*. Two other monographs, *The Biology of Human Starvation* (208), and *Nutrition Surveys* will also be issued.

STUDIES ON WARTIME UNDERNUTRITION

In recent years prison and concentration camps, occupied areas, and the total dislocation of war in various countries provided countless victims of starvation and malnutrition of all types and degrees. The observations, measurements, and tests made on these unfortunate people constitute a major contribution to the advancing knowledge of nutrition. Many reports are still to be published but those made available in the past two years represent a large and valuable literature.

In 1946 there was published at Warsaw an extraordinary volume containing the findings, including many experimental studies, on the large population of Jews confined to the Ghetto for nearly two years before final "liquidation" in 1942 (17). A medical research team made up of incarcerated doctors and scientists worked in two hospitals within the Ghetto. As elsewhere under famine conditions in Europe, the overwhelming importance of calories was soon appreciated and signs of specific deficiencies were few and unimportant.

Equally starved persons from the concentration camps were studied in detail on release by Lamy *et al.* (226) in a French hospital and by Hottinger *et al.* (175) in Swiss hospitals. The patients studied by Lamy *et al.* were chosen to represent "pure" starvation but the majority were febrile and had tachycardia and albuminuria, so other serious complications must have been present. Almost half of the patients who reached Switzerland had tuberculosis.

Both of these groups were studied in early rehabilitation, that is after the active phase of starvation. In the Netherlands East Indies extensive studies on malnourished persons from the internment camps (283) covered a slightly later phase of refeeding.

The immediate condition of 771 victims from German prison camps was studied by Debray *et al.* (83). Kars (193) analyzed some findings on a large number of repatriated political prisoners. Studies during active severe undernutrition were made over a prolonged period in a French insane asylum by Gounelle and his colleagues (134, 135, 136) and in a Belgian prison by Simonart (333). Data from the siege of Leningrad pertain only to persons seen in the hospitals from 1941 to 1943, but they exhibit many points of interest, some of which have been summarized by Brozek *et al.* (52, 54).

The findings in the Netherlands during the famine of 1944-45 and in the preceding years of more limited undernutrition have been assembled in several places (45, 94, 122, 301). For Belgium, Brull *et al.* (56) provided a monograph reporting the studies carried on at Liège throughout the period of the German occupation. The papers presented at a symposium held at Liège after the war have been published under the editorship of Bigwood (38). It contains a large number of useful papers relating to the nutritional situation in Belgium, France, and the Netherlands, with some data on England and other areas of Europe. The essential uniformity of the findings is striking—moderate anemia and edema with only slight hypoproteinemia, unexplained diarrhea and polyuria without evidence of renal disorder, extremely little evidence for vitamin deficiencies, improved diabetes, and increased tuberculosis and gastrointestinal disorders.

At the end of the war, Beattie and his colleagues carried out some detailed studies on severely undernourished people in the Netherlands and in Germany; it was possible to make some experiments under controlled conditions in Germany. At much the same time the Oxford Nutrition Survey, under Sinclair, was active in the Netherlands and Germany; some of the results are available (338, 339), but, presumably, a fuller report is still to come. Later, a special study was set up by the British at Wuppertal in the Ruhr but the reports are still awaited (128). From Japan the only data available are those provided by the U. S. Strategic Bombing Survey (376). The data and interpretations leave much

to be desired but it is clear that Japan, though undernourished, fared far better than did the areas occupied by the Axis. The Minnesota Starvation Experiment, which afforded many parallels to European famine, began in 1944 and continued through part of 1946. It is briefly mentioned below in the section on Experimental Dietary Restriction.

A number of reports have been published on studies made in prison and concentration camps (194, 358); one of the best is that of Leyton (234). The largest number of such reports is from the Japanese camps where vitamin deficiencies and neurological manifestations dominated the picture; these are discussed in several sections below. For various reasons there are no real reports on undernutrition published by nationals of the former Axis powers. Fleisch (109, 110) has provided a detailed analysis of the war-time diet and its consequences in Switzerland. Dietary surveys in Norway for 1942-45 are summarized by Strøm (361). A general treatise on the "Geography of Hunger" has been published by de Castro (60). Nutritional problems with domestic animals in war-time Europe have been discussed (51, 123, 195, 237).

EXPERIMENTAL DIETARY RESTRICTION IN MAN

During the war the voluntary subjection of conscientious objectors to dietary restriction allowed a number of important nutritional studies to be made. The results of the experiment on vitamin A restriction at Sheffield, England, are not yet reported in full but they are obviously of great importance (68, 247). Sixteen "normal" individuals were on a diet devoid of vitamin A, and containing about 70 I. U. of carotene daily for up to two years. No thrombopenia or changes in the sclera or cornea occurred but three individuals developed reduced dark adaptation after 14, 17 and 22 months. Where follicular hyperkeratosis was seen it was not specially related to the state of vitamin A nutrition; this finding was independently confirmed (341). Somewhat similar studies on ascorbic acid deficiency are noted in a separate section (see p. 516).

An experiment at Chicago on seven young men involved 36 weeks on a diet adequate in calories but providing only 45 gm. of protein and various severe limitations of the members of the vitamin B complex (35). The restricted period was preceded by 12 weeks' control period on a fully adequate diet and was followed

by three weeks of rehabilitation studies. Incontrovertible stigmata of deficiency were not observed but there were various complaints as well as objective alterations of personality and physical performance. Repeated applications of the Minnesota Multiphasic Personality Inventory showed increasing depression, hypochondriasis, and hysteria during the period of restriction, with a rapid return to the previous normal scores on a full diet (162). During restriction the excretion of creatinine gradually declined and was considered to be somewhat related to the state of physical fitness; in the control period each man had a well-characterized individual level of creatinine excretion (124). It was observed that the urinary excretion of vitamins changed rapidly in response to temporary changes in intake without parallel changes in performance, general health or basic nutritional status (34). Other experiments at the same laboratory confirmed previous findings at Minneapolis (67).

The maintenance of normal men for many months on rigidly controlled diets with fixed different levels of thiamine intake at Minneapolis allowed a rigorous analysis of the individual characteristics in excretion at intakes from 0.6 to 16 mg. daily (262, 263). Following a change of 1 mg. in the daily thiamine intake, about six weeks were required for full stabilization of the urinary excretion at the new plateau. At all levels of intake, individual differences were large and consistent. At low intake levels the excretion of pyramin rather than thiamine itself appeared to be more useful for appraising thiamine nutrition. Salcedo *et al.* (316) have shown the limited value of the fasting hour thiamine excretion in a most interesting study in an area of endemic beriberi in the Philippines. Other valuable data on thiamine excretion have appeared (41, 125, 173).

Some of the results from the Minnesota Starvation Experiment have been published (53, 119, 164, 202, 203, 205, 206, 207, 209, 210, 228, 264, 323, 334, 336, 367). In this project 32 normal young men were studied for three months on a fully adequate diet, averaging 3,450 kcal. daily, for six months on an average intake of 1,560 kcal. on which they lost a fourth of the original body weight and became edematous, and for 12 weeks on controlled rehabilitation diets. Studies were continued for a year following the end of semistarvation. A large number of objective tests and measurements, repeated throughout, covered anthropometric, biochemical,

physiological, and psychological details. The semistarvation and early rehabilitation diets followed the European famine pattern—potatoes, whole wheat bread, cabbage, and turnips, with very little meat or dairy products. As in Europe, the caloric factor proved by far the most important in the diet. Body weight and many other changes were restored to normal in five months of refeeding but the prestarvation state was not recovered in all respects until some months later.

For a number of years controlled studies on vitamin restriction have been under way at the Elgin State Hospital and a definitive report is now available (173). Wet beriberi as well as other evidences of thiamine deficiency were produced. With a restriction period of three years, a total intake of 0.4 mg. of thiamine per day is below the minimal requirement for relatively inactive elderly men. Evidence for the result of deficiency in other members of the B complex was inconclusive.

NUTRITIONAL SURVEYS

Nutritional surveys are conducted on the basis of estimating intake of nutrients or physical evaluation of nutritional status or both; the necessity for the combination is increasingly apparent. Bean (25) has analyzed the factor of subjectivity in physical examination. Recent discussions of methods and interpretations (2, 47, 79, 257, 294, 319, 362) deserve study. Surveys of the nutritional situation in Newfoundland (75), Nova Scotia (319), and the Gaspé peninsula (21), continue to exemplify the differences in methods and interpretations which cause sociological controversy over this aspect of nutrition. Sinclair (341) has critically discussed problems of physical appraisals in surveys.

Surveys in Great Britain continue to indicate a relative satisfactory nutritional status judged from physical examinations (3, 240, 265, 330). The great differences in caloric intake between old people (304) and adolescents (394) suggest that the age factor is underestimated in most tables of "requirements." Surveys among pregnant women in Great Britain generally fail to disclose signs of nutritional deficiency (167, 404) or any relation between income and nutritional status (404). Careful studies of food intake of 111 pregnant women showed not one who attained the recommended allowances of the National Research Council (U. S.), but clinically they were well nourished (167).

An elaborate study in the Netherlands in 1945-46 indicated no close correlation between plasma protein level and either diet or other indications of nutritional status (224). In India, studies of children showed no correlation between the general state of nutrition and the incidence of signs of specific deficiency (phrynoderma, angular stomatitis, xerophthalmia, and so on) (267); a rather similar dissociation between general status and specific signs appeared from statistical analysis of data in England (2). Surveys are reported from China (196, 386, 387), British prisons (303), British Colonies (297, 298), Italy (259, 290), France (372) Canada (48, 275, 293), India (20, 328), Greece (6, 62), Austria (81), Mexico (12), the Ukraine (117), Tasmania (286), Australia (281) Belgium (24, 285) Holland (122, 307, 355), and the United States (65, 395); with some exceptions these are concentrated mainly on either food intake or the apparent nutritional status.

HUNGER AND APPETITE

All reports agree that hunger largely disappears in a few days of fasting but that hunger persists and increases in semistarvation until the terminal stage is approached; anorexia in famine victims makes a bad prognosis (207). In refeeding after famine, enormous amounts of food (6,000 to 12,000 kcal. daily) are required to satisfy appetite and there is a tendency to obesity on an *ad lib.* diet (207, 280).

Vagotomy of both men and dogs appears to abolish hunger contractions of the stomach but does not abolish hunger itself and insulin still provokes a sense of hunger and causes an increase in food consumption (143, 144). Amphetamine (benzedrine) results in weight loss largely by reducing appetite (156). Various aminopropane compounds are effective anorexigenic compounds in the reduction of obesity (398).

EFFECTS OF SINGLE MEALS

Experiments with small isocaloric test meals indicated no significant differences in rate of gastric emptying or final emptying time within all nutritionally reasonable limits of variation in the proportions of fat, protein and carbohydrate (163). With very large (1,500 kcal.) meals, however, added fat delayed evacuation (14).

In strenuous visual work, four types of preparatory meal (no

meal, balanced meal, high fat, high carbohydrate) were compared in controlled experiments on normal men (335). A battery of criteria including performance, sensation, retinal functions and standard ophthalmological tests, indicated no type of meal (including no meal) to be superior in all respects. In strenuous physical work tests on the bicycle ergometer, Haldi & Wynn (152) found no specific advantage in a previous high carbohydrate meal and no relation between the blood sugar level and the capacity for work. Many details of the electrocardiogram are altered by an ordinary meal, the composition of which is unimportant; the meal tends to reveal or accentuate latent abnormalities and may be useful in diagnosis (336, 337).

NUTRITION AND BASAL METABOLISM

The perennial argument about the "best" way to express the basal metabolic rate continues with increasing objection to body surface as the basis (26, 126, 215). When standards based on the body surface are used, the basal metabolism is generally found to be depressed in undernutrition more or less in proportion to the degree of weight loss (26, 111, 137, 192, 207, 209, 333). In one study on 49 persons with famine edema the basal metabolism was only slightly below the Harris-Benedict standards but on re-feeding it rose above those standards (189). Measurements on 84 men and 34 women in rationed Germany indicated low rates, even in those women who had body weights close to normal (192).

Kirk & Kvorning (213) analyzed the symptoms presented in 308 patients with basal metabolic rates of -12 per cent or below, without regard to cause of the hypometabolism. Prominent among the associated symptoms were bradycardia, chilliness, fatigue, somnolence, hoarseness, and falling hair. All except the last two symptoms are universally characteristic of semistarvation and many starved people have complained that their hair fell out. Even the hoarseness recalls this complaint in oriental beriberi (see 350).

It is clear that the total basal metabolism of the body falls as undernutrition progresses, but the question remains as to whether there is a corresponding change in the basal energy turnover of the metabolizing tissues. Calculating the active metabolic mass of the body as the body weight less the fat, extracellular fluid and bone mineral, it appears that most or all of the reduction

in total metabolism is accounted for; i.e. the basal oxygen usage per unit weight of "active" tissue is little changed (26, 207). Obviously, there are many interesting possibilities with this method of calculation.

CALORIC REQUIREMENTS

The practical experiences of World War II have stimulated criticism of the National Research Council *Recommended Daily Allowances*, notably with regard to calories (e.g., 53). The new revision (282) of the Allowances makes minor concessions by slight reductions in the recommendations for sedentary man and woman, for moderately active woman, and for pregnancy. A footnote to the Table admits the necessity for adjustment to individual needs but states that the kilocalorie allowances "represent group averages." Even so, all the recommended caloric figures still seem high and undoubtedly oversimplify the question by omitting any reference to the effects of age (of adults) and of climate.

Old people in London apparently get along well on averages (for small groups) of 1,434 to 2,160 kcal. for men and 1,409 to 1,579 kcal. for women (304). An elaborate and detailed analysis of nutrition in Switzerland during the war led to the conclusion that the caloric "requirements" of the League of Nations are excessive; instead of the pre-war 2,400 kcal. daily for a man with no special manual work, an average of 2,160 kcal. seemed to be reflected in a very favorable health record (110). Moderate caloric reductions in Great Britain were likewise considered to have been beneficial or at least not detrimental. From the vantage point of the Ministry of Health, Magee (241) doubts whether a significant number of hard workers anywhere "require much more than 3,500 Calories 7 days a week." In the Royal Canadian Air Force in 1943-45, the daily food eaten in the messes averages 2,869 kcal. per man; presumably something like 200 to 400 kcal. in addition should be allowed for food eaten outside the messes (48). In Mississippi, data from farm laboring families indicated averages of 2,219 and 1,555 kcal. for white men and women, respectively, and 1,645 and 1,173 kcal. for colored men and women (403). These people were clinically not underfed.

In Italy studies were made on the actual rate of energy expenditure at some of the hardest types of labor. At hard work in the rice fields and in hand mowing, about 335 kcal. per hour were expended, from which it was concluded that about 3,600 kcal.

would cover the day, including eight hours of this work (58, 138). Hand labor in the mines was more severe and called for something like 350 to 400 kcal. per hour, resulting in 24-hour estimates of 4,400 to 5,200 kcal. (139). Such estimates are apt to err in assuming that a miner is always actively mining, a mower swinging the scythe, and so on, nor can the estimates be extrapolated properly to a yearly or even weekly basis. Where actual food consumption is studied on a longer time basis, the result is almost invariably much less. Means for two periods of four weeks each (summer and winter) in Sardinia indicated balances at 2,756 kcal. for farm laborers and 2,609 kcal. for shepherds (290).

In North China, studies on 2,002 high school students from 1934-38 indicated daily intake averages for summer and winter of 2,930 and 3,110 kcal., respectively, for boys and 2,120 and 2,190 for girls; the caloric supplies were fairly abundant (before the war) but 6 per cent showed some signs of malnutrition (196). Measurements are reported for the energy cost of simple activities of American children in a closed circuit metabolism chamber (365, 366). Before the war in China the Army medical students had an average of 2,936 kcal., but during 1938-45 this fell to 2,689 kcal.; the pre-war level was not associated with signs of caloric undernutrition (387). Ten surveys in the Chinese Army from 1936 to 1945 gave a range of averages from 2,176 to 3,153 kcal., with conclusions that 67 per cent of the rations were inadequate in calories (386). Dock laborers in Belgium doing heavy work in winter on an intake of 2,460 kcal. gained an average of 1.3 kg. in 10 days when the total diet was increased to about 3,700 kcal. (342).

An average intake of 2,284 kcal. in 15 maternity hospitals in England was 262 kcal. below a "target" diet (74) but there were no data to show that the intake was really inadequate in calories. A careful survey in England of 111 pregnant women without any stigmata of nutritional deficiency showed average intakes for the second trimester of $2,400 \pm 430$ kcal.; this group had a good record for their pregnancies, deliveries and post partum state (167). Widows (394) has reported in full on 1,028 British children studied in 1935-39; most of these children were from the middle class and lived at home. On the average 15-year-old boys had 3,400 kcal., and girls of the same age had 2,588 kcal.; the height-weight data were good.

All reports continue to show wide individual variations in

caloric intakes and, apparently, in requirements for balance at satisfactory body weights (e.g., 394). It is probably less hazardous to estimate proper calories for a country (49) than for workers in an industry (118). Climatic differences have a very large effect (183); it would seem ridiculous to apply the same scale to Minneapolis and New Orleans or even to the same city in winter and summer. In any case, data from animal studies continue to show that, if longevity is the criterion, caloric restriction should be practiced (251, 309). Good standards for body weight or fatness would seem more likely to promote proper caloric nutrition than deceptively simple tables of caloric "needs."

PREGNANCY AND FETAL DEVELOPMENT

Various animal experiments have proved that cunningly contrived malnutrition can disturb the course of pregnancy and fetal development, but the relevance of these facts to actual human nutrition is not yet very clear. In the Netherlands the complications of eclampsia and the toxemias of pregnancy were minimal during famine (106, 344). A lack of correlation between maternal diet in famine and the incidence of congenital defects has been noted (343). In the famine of the siege of Leningrad it has been reported that there was an increased proportion of premature births and stillbirths (16), but Smith (344) could find no valid evidence for such a situation in the famine of Western Holland.

A relation between the size of the newborn and the quality and quantity of the diet emerges rather clearly from data collected in areas of food shortage. In actual famine in Greece, average decreases in birth weight of 100 to 250 gm. occurred (377). Much greater reductions were reported for famine in the Netherlands (45), but a detailed analysis showed that the average changes were of the order of 300 to 400 gm. at the extreme and that the effect of maternal starvation is most pronounced in the last trimester of pregnancy (343, 344). The data from famine in Leningrad are rather similar but somewhat more pronounced declines (400 to 500 gm.) were reported (16). Even greater declines were claimed for Vienna at the end of World War II (178) but the evidence is poor. Considerable changes in general nutrition were not reflected in birth weight in Chengtu, West China (230), and the total evidence indicates that birth weight is not significantly altered until the mothers are seriously and obviously underfed.

GROWTH OF CHILDREN

Quantitative evidence on the effect of nutrition on the growth of children continues to be surprisingly meager. One careful study merely "suggested" that the retarded growth in some thin children was due primarily to caloric malnutrition (364). The reported changes in growth rate in France in World War II are small (159, 371) and even in the Netherlands the food shortages and famine produced only slight to moderate reductions in growth (150, 190). In Greece, measurements on 100,000 children showed the majority to be underweight in 1942-44 (377). At the end of the war there were no marked evidences of depressed growth of children in Italy (259) or Vienna (302). All authorities agree that the reduction of growth in length in underfeeding is much less than the reduction in weight growth (*op. cit.*, 50). One reason for the slight effects of food shortages on the growth of European children in World War II to be seen in the great efforts made to give them a maximum share of the available foodstuffs. During refeeding after famine, Dutch children grew rapidly (150). Sinclair (340) reminds us that the virtues of a rapid growth rate in children are not fully proved.

NITROGEN METABOLISM AND NUTRITION

The general aspects of protein requirements and deficiency have been discussed (30, 266). The relation between protein nutrition and other nutrients has been reviewed (89). Problems of nitrogen metabolism with regard to repletion after protein depletion continue in interest (7, 171, 274, 291). Benditt *et al.* (29) argued for the desirability of giving priority to high protein feeding in rehabilitation following protein depletion or general undernutrition. Homburger (171) properly pointed out the limitations to evaluating protein status on the basis of simple measurements of plasma protein concentration. Robschey-Robbins *et al.* (312b) suggests that the blood proteins may be preferentially formed at the expense of tissue proteins. However, analyses of samples of liver and blood plasma in surgical operations showed these to be closely correlated (232). In the dog, different amino acid mixtures are indicated for most efficient production of hemoglobin on the one hand and of plasma proteins on the other (312b).

Peters (291) favors attempts to reduce protein wastage in

the body in disease and injury, but is not enthusiastic about heroic efforts at protein administration. F. D. Moore (274) expressed skepticism over the real value of forcing nitrogen intake. Keeton *et al.* (199) showed that early ambulation reduced the negative nitrogen balance following surgery. Dietrick *et al.* (90) report in detail on four normal young men who were immobilized for five to seven weeks and received the same diet as in a control period with normal activity. It is puzzling that there were only trifling changes in body weight in spite of the great change in the physical regimen; it is possible that the heavy plaster casts did not invite as much repose as immobility. In any case, confirming other studies, there was a substantial increase in the excretion of nitrogen and calcium, with an average total loss of 53.6 gm. of body nitrogen. Howard *et al.* (177) have reported their balance studies on two normal young men in bed rest. There is room for debate from these findings in bed rest as to what constitutes a proper diet in illness and convalescence; the relation between nitrogen balance and bodily function is not clear (274, 291).

Interest in the use of testosterone to prevent or reverse the negative nitrogen balance in injured states continues without impressive new evidence (1, 186, 253, 261). Relations between hormones and protein metabolism are reviewed by White (392). Many papers have appeared on various amino acid and protein preparations and regimens to increase nitrogen retention (e.g., 1, 100, 103, 261, 308, 349, 378b, 389). The general problem in surgery has been reviewed (238). Keeton *et al.* (199), working with surgical patients, maintained a positive nitrogen balance at all times with a diet providing twice the basal caloric needs and including 2.6 gm. of protein per kg. of body weight. Positive balance for the day of surgery and five succeeding days resulted from a diet providing 120 per cent of basal caloric needs with 2.7 gm. of protein per kg. A high caloric diet alone was also effective in promoting nitrogen retention. All of these regimens reduced the urinary urobilinogen. Extra vitamins and methionine had no effect.

The hope that methionine would have peculiar virtues in increasing the efficiency of human nitrogen metabolism seems to have died with the publication of clear evidence that methionine is less critical for men than for dogs and rats (8, 57, 71, 184). Human methionine excretion is relatively independent of intake (368). A five-year review of experience with a regimen of choline,

methionine, and low fat in hepatic cirrhosis indicated little, if any, benefit (382).

New experiments comparing various proteins in human nutrition continue to demonstrate that differences in quality are less clear than indicated by the textbooks. Mueller & Cox (278) found no difference between casein and lactalbumin in four normal adults. In experiments on six persons the quality of the protein in milk and rice mixtures was only slightly greater (though significantly) than in mixtures of rice and pulses (269). Comparative studies on men, dogs, and rats on the same diets indicated definitely greater digestibility and slightly greater biological values of the absorbed proteins in man; the differences between man and dog were greater than between man and rat but there were real differences between all three species (160). Feeding experiments for 24 weeks on 801 Indian Army recruits indicated the value of adding meat and milk to the regular ration for those men showing deficiency signs (380). Mack *et al.* (239) studied children at two institutions and concluded that meat 10 times a week was preferable to meat twice a week. In the subarctic, where there is a very high intake of animal protein, there is no rheumatic fever, chorea, Bright's disease or polyarthritis; in 14 years with about 400 births a year, there was only one case of eclampsia (99). Protein nutrition in pediatrics and in pregnancy has been reviewed by Levine (233). A high protein, low calorie diet is considered desirable, with regard to the toxemias of pregnancy (236). Anthropometric data from Sardinia are interpreted to favor a high intake of animal protein (290). Experiments with poorly fed workers in Brussels in the winter of 1945 were considered to prove the value of a high protein dietary supplement but there was no relation between nitrogen balance and functional improvement in individuals (342).

The general significance of plasma proteins and protein metabolites is ably reviewed by Metcalf & Stare (260) and by Sahyun (313). New data on plasma protein standards have been published (39, 44, 270). There is a direct relation between protein intake and blood urea concentration in normal man (4). Experiments on dogs failed to show any evidence for a "premortal rise" in nitrogen excretion (390). Balance studies on man indicated that wheat flakes and exploded wheat are inferior to unprocessed whole wheat in maintaining nitrogen equilibrium but similar processing of corn and oats did not produce this change (220).

NUTRITIONAL EDEMA

Nutritional edema is discussed in almost all recent papers on severe undernutrition. It now appears that four types, which may occur in any combination, are to be distinguished: simple famine edema, hypoproteinemic edema, neurogenic edema, and congestive failure edema. While hypoproteinemia is often observed in edematous starved persons, it is by no means universal (17, 27, 59, 122, 135, 140, 145, 203, 207, 210, 225, 226, 276, 338, 339). The absence of edema in some of the most severely starved men with extreme hypoproteinemia is interesting (107, 300).

The fact that some correlation is frequently found between the plasma protein concentration and the extent of edema in famine, tempted some workers to explain all nutritional edema on this basis (42, 87, 137, 222, 292). Sinclair (338, 339) has emphasized the error of such reasoning. Though hypoproteinemia can be contributory, the numerous cases of edema with normoproteinemia are of more interest. The suggestion that the colloid osmotic pressure *in vivo* may be reduced through leakage at the capillary wall (59) is untenable in view of the very low protein concentration in the edema fluid (135, 137, 207, 210). The theory that the molecular size of the plasma proteins in starvation is abnormally large is discussed below; after careful consideration, it is doubted that this explanation could be sufficient (207, 225, 226, 339).

Controlled studies on simple famine edema without hypoproteinemia show that the ratio of extracellular fluid (thiocyanate space) to body weight is much increased but that the absolute amount of extracellular fluid is little changed from the pre-starvation level (27, 164, 207, 210) and other data support this conclusion (87, 201, 363, 385). Hence, simple famine edema involves little or no new accumulation of fluid and its clinical appearance merely reflects a change owing to a loss of cellular mass (164, 207, 210). Beattie & Herbert (27) confirm this fact and term the situation "isohydric" edema. Clinical edema appears when there is a relative excess of extracellular fluid amounting to about 10 per cent of the body fluid (27, 164, 207, 210).

Extreme edema and ascites are most often seen in persons who have suffered prolonged semistarvation and who also have marked hypoproteinemia and anemia (46, 222); these cases would seem to represent the combined effect of emaciation (simple famine

edema) and hypoproteinemia. The association of edema with diarrhea and enteritis continues to be noted without explanation (31, 50, 292, 296). The edematous men in the Minnesota Experiment had no diarrhea (207). Epidemic dropsy in India (Bengal edema) appears to be caused by the toxic action of mustard seed (Argemone) oil which is sometimes consumed in times of food shortage (268); cardiac damage may be involved. A gross abnormality of the chloride content of the skin in famine edema is reported (9).

Normally in famine edema the venous pressure is low (137, 208, 209, 210), but in refeeding on a full diet the pressure rises and may reach the congestive failure level. In such cases edema may reappear on the new basis of cardiac failure (209). Return, exacerbation, or first appearance of edema during refeeding after semistarvation is not uncommon (87, 210, 352).

Unilateral or otherwise localized edema sometimes appears in severely malnourished persons with nutritional neuropathies (350). This condition, which is occasionally very striking, may be termed nutritional neurogenic edema. The mechanism has not been studied. Simonart (332, 333) attempted to explain the edema studied in a Belgian prison as the result of thiamine deficiency; the data are unconvincing and in some respects palpably erroneous. Heart failure seems to be the basic explanation of beriberi edema but oriental wet beriberi may often involve simple famine edema and hypoproteinemia as well (283).

Brull (55) continues to suggest a renal basis in famine edema without impressive evidence except for the failure of explanations based on hypoproteinemia, thiamine deficiency, or heart failure. Renal damage was seen by Lamy *et al.* (226) but the starved men were febrile, had tachycardia, and showed albuminuria and, hence, were entirely atypical. Blood and urine analyses in famine edema generally disclose no renal insufficiency (50, 111, 172, 272, 338, 352). Polyuria and nocturia are generally noted in famine but would seem to be explained by watery diets and polydipsia (72, 146, 207, 226, 234, 283). However, we may agree with Sinclair (339) that renal function should be studied further.

Except for the psychological state, there seems to be no essential difference between anorexia nervosa and ordinary semistarvation such as seen in famine. Edema is not rare but is less common among anorexia nervosa patients because many of these have an

aversion to the ingestion of anything, including water, and hence tend toward dehydration (32, 33). In edematous patients with tropical sprue the total serum proteins may be normal; in early treatment there is often a fall in the protein concentration and an extension of the edema (354).

PHYSICAL CHEMISTRY OF PLASMA PROTEINS IN FAMINE EDEMA

The occurrence of edema in large numbers of undernourished persons who exhibit no signs of cardiac or renal disability or of capillary damage and who have apparently fairly normal concentrations of proteins in the plasma, has stimulated suggestions that the molecular size of the proteins may be abnormal in starvation. Govaerts (137) measured colloid osmotic pressures in serums from 38 patients, mostly elderly men, and obtained values generally lower than could be calculated by his formula from chemical analysis. The formula used, however, is not derivable from physicochemical theory.

Lamy *et al.* (225, 226) also obtained from colloid osmotic pressure measurements some suggestions that the mean size of the protein molecules may be slightly increased in starvation and there have been other hints to this effect (338). Support for this view was obtained from ultracentrifuge measurements which indicated that something less than 10 per cent of the globulins may be present as macromolecules of perhaps six times the ordinary size (225, 226). Though these latter observations suggest a reduced osmotic activity of the proteins, the effect would be small and could not account for the edema. Peculiarities in the precipitation behavior of plasma proteins have been claimed in famine edema (165). It is reported that the ratio of arginine to lysine, which in the normal total plasma protein is 1 to 1.85 or 1 to 1.90, may be quite different (with an extreme of 1 to 4.4) in persons with famine edema and a similar change was produced in a dog subjected to severe protein undernutrition (112). No qualitative abnormalities in the plasma proteins were found in eight patients with nutritional hypoproteinemia; electrophoretic and immunological methods were used (36). Electrophoretic studies on the serum from dogs depleted in protein and then refed are available (64); the significance is not clear. The assembled evidence indicates that precise and detailed studies of the proteins in semistarvation are greatly needed.

CARBOHYDRATE METABOLISM IN UNDERNUTRITION

The favorable effect of food restriction on diabetes mellitus is noted below ("Noninfectious Disease and Nutrition, p. 507). The basal blood sugar concentrations in 153 semistarved Russians tended to be slightly low, particularly in comparison with 51 better fed British prisoners in the same camp; the lowest values were in the most severely starved men (234). Persons with famine edema generally show low blood sugar concentrations (42, 146). Gounelle (134, 135, 136, 245) frequently observed hypoglycemia and reported a number of deaths in hypoglycemic coma among severely starved men.

At Warsaw, moderate to severe hypoglycemia was the rule in the semistarved population; most values were around 60 mg. per 100 cc. (normal 80 to 120 mg.) and a low of 23 mg. was recorded (50, 111). Both epinephrine and insulin provoked very small and delayed responses in the blood sugar; 50 gm. of glucose by mouth produced only a slight elevation, likewise delayed, followed by a more profound hypoglycemia. In these experiments at Warsaw paradoxical arterial-venous gradients in blood sugar were often seen following epinephrine or insulin (50, 111). At Warsaw, as elsewhere, it was noted that extraordinarily low values of blood sugar in starving people were often unattended by any special symptoms. Lamy *et al.* (226) also observed a lack of blood sugar response to epinephrine.

Further details on carbohydrate metabolism in undernutrition are scanty. Simonart's (333) data on the respiratory quotient and blood pyruvate are useless because of obvious technical faults. More acceptable studies on pyruvic acid in the blood show that this tends to be slightly high in semistarvation, even when there is no evidence for any thiamine deficiency (283, 338). In contrast to fasting, ketosis and ketonuria ordinarily do not occur in semistarvation (226, 283).

THE SKIN IN FAMINE

Reports continue in agreement that the skin of severely undernourished people is generally rough, cold, dry, and thin, with a marked tendency to what is variously labelled, without very clear distinction, as folliculosis, keratosis pilaris, hyperkeratosis, or "permanent goose flesh"; there is no evidence that a deficiency of vitamin A is involved (17, 175, 226, 247, 255, 283). The problem is

important because of the general tendency to diagnose vitamin A deficiency whenever such skin conditions are seen. Vitamin A studies and experiments on 215 children with folliculosis and 179 without this condition, none of whom was starved, failed to indicate any relation between vitamin A and folliculosis (218).

In the 492 cases of "pure" starvation death studied at Warsaw there was a marked brownish pigmentation of the skin in 13.8 per cent of the bodies (356). A tendency to this type of pigmentation, appearing first in flecks or patches and later becoming confluent, must now be recognized as a fairly common result of severe caloric undernutrition. It is sometimes localized around old scars but occasionally may affect the entire body (50). In one series of undernourished patients in Vienna there were 80 cases, mostly women who sought cosmetic relief (19). In Brussels women between 40 and 50 years of age were the most frequent among the patients seeking dermatological consultation because of this pigmentation (255). The condition was fairly common in some of the Japanese concentration camps in the Netherlands East Indies (283); in the prisoners taken at Hong Kong (December 25, 1941) this "famine pigmentation" was a part of the typical description (72). Famine pigmentation is clinically, and probably metabolically, distinct from the pigmentations of pellagra and of Addison's disease. Apparently a different condition, possibly related to niacin deficiency, was called skin pellagra at Singapore (227).

HUNGER OSTEOPATHY

As was the case in the first World War, the widespread food shortages of World War II produced a steady increase in the incidence of osteopathies, mostly of the demineralization type generally termed osteomalacia. Data from Paris illustrate the trend: 3 cases in 1940, 5 in 1941, 8 in 1942, 24 in 1943 and 51 in the first 6 months of 1945 (88). The majority of cases were like the 20 patients reported in one series—women from 50 to 70 years of age who had lost 15 to 20 kg. on a diet mainly composed of bread, legumes, little meat and no milk (88, 145). Pompen *et al.* (301) have discussed the problem in detail for 24 typical cases in the Netherlands; their patients were considered to be generally undernourished, subsisting on a low calcium intake and deprived of vitamin D and sunlight.

In the Warsaw Ghetto a tendency to spontaneous fracture and

delayed bone reunion was noted (111). Roentgenological evidence of decalcification was seen in German concentration camp victims (226), and in prisoners in Japanese camps (283), but the incidence of definite osteomalacia in semistarved populations is unknown; it obviously affects only a small fraction of the starving. It is of interest that severely undernourished children seem to be relatively immune to the disorders of calcification affecting the adults, and rickets may be decreased (50) or, at most, only moderately increased (235).

The etiological factors of calcium intake and vitamin D in hunger osteomalacia are not clear. The majority opinion is that calcium alone is useless but that a good diet and large doses of vitamin D produces fairly prompt healing and recalcification (88, 145, 283, 301).

NUTRITION AND INFECTION

There are few questions more complex than that of the relation between the nutritional state and susceptibility and resistance to infectious disease. Broad generalizations based on "common sense" and the historical association of famine and pestilence, die hard, but more critical analyses are appearing (258, 289, 324). Understanding is not bettered by unjustified claims about the situation in Japan (376).

New evidence appears from Cannon's laboratory that antibody formation is depressed in extreme protein depletion (400). In view of concern as to the effect of protein nutrition on infection, it is interesting that rats and mice are reported to have a decreased resistance to *Salmonella* infection while subsisting on a low protein and low caloric diet but that resistance may be improved by the addition of either calories or proteins (147, 148). However, in elaborate studies on *Salmonella* infection in rats, Metcalf *et al.* (258) found no effect of a low protein diet on the response to the infection, though there were changes in the plasma proteins.

Studies on virus infections in malnourished animals continue to demonstrate complications. Low protein diets may delay the onset of symptoms after inoculation with poliomyelitis virus without changing the ultimate mortality rate (188). Thiamine deficiency affects the course of equine encephalitis in mice without greatly affecting mortality but inanition alone has no effect (197). In mice inoculated with the virus of swine influenza the lowest mortality

was on a protein-deficient diet; addition of methionine resulted in higher mortality and the least resistance was observed in the animals on the "best" diet with abundant proteins (351). Interest in gamma globulins continues without providing proof that this fraction is peculiarly sensitive to nutrition or that resistance to infection may be safely inferred from the level of gamma globulins in the blood (219, 346).

Recent experience with infectious disease in severely undernourished populations is of much interest. Pyodermias and furunculosis were extremely common in underfed Europe but absence of soap, hot water, and the general condition of extremely bad personal hygiene seem to offer sufficient explanation (17, 50, 133, 174, 201, 375). In general, contagious diseases, with the exception of typhus and tuberculosis, caused surprisingly little trouble in the famine areas of World War II.

At Warsaw measles and meningitis were mild in their course and reduced in incidence in the large population of starving children; the better fed children fared worse from these contagions (50). Rheumatic fever almost disappeared in Warsaw in 1940-42 (*ibid.*). In Vienna, however, it was believed that measles were unusually virulent and the percentage mortality in diphtheria was doubled in 1945 when undernutrition was general (221).

During and after World War II diarrheal conditions were severe and widespread among most population groups suffering from malnutrition. Apart from the uncertain factor of bad sanitation, there is also the question as to whether enteric infections were involved; in the great majority of cases pathogens were not demonstrated in the stools (17, 201, 283). The absence of complications from infection in grossly malnourished persons after surgery under the crudest conditions of attempted asepsis, was sometimes notable (133).

The course and clinical character of infectious disease in man is altered by severe undernutrition. A lack of febrile response has been reported many times (e.g., 17, 283). The explanation for various discrepancies in the literature about fever in starving men may be found in the fact that the starving man is relatively poikilothermic and nonreactive (17, 50).

NONINFECTIOUS DISEASE AND NUTRITION

Information is accumulating on the effect of severe under-

nutrition on the incidence and course of noninfectious diseases. New reports (17, 182, 191, 242, 283, 379) bear out previous indications that the incidence and mortality of diabetes mellitus is markedly and progressively reduced in populations on short rations though management of the most severe cases poses problems (235). Evidence of abnormality in carbohydrate metabolism in the obese continues to be reported (130).

At routine necropsy there are few atheromata in emaciated bodies but the incidence of atherosclerosis is high in the obese (396, 397). A remarkable reduction in cardiovascular deaths during the siege of Leningrad (54) was followed by a still more remarkable epidemic of hypertension after the siege was broken and better diets became available (52). Some cases of poststarvation hypertension were seen during refeeding in Malaya and Java (283, 352). Another bad consequence in the refeeding period after famine is reported to be a great increase in gallstones (320). An interesting relation between gallstone incidence and food habits in Finland has been reported (104).

Undernutrition in the Netherlands is stated to have been associated with a reduction in eclampsia with a sharp rise in cases during the subsequent refeeding period (106). At Warsaw, rheumatic fever practically disappeared from the large population starving in the ghetto (50), and there was surprise that there were indications of amyloidosis in only 6 out of 3,282 necropsies (356).

Peptic ulcer seems to increase in most areas of wartime food shortage, and emphasis shifts from the duodenum to the stomach (145, 168, 277, 401). In the most severely starved persons, however, peptic ulcer is not found; this is understandable in view of the hypoacidity or anacidity customary in such cases (111, 168, 333). In severe famine conditions the mortality from cancer is not increased but there is no real evidence that the incidence and progression of neoplasms is reduced (94, 377).

Allergic and hyperreactive conditions in general, including eczema, psoriasis, and asthma, may be decreased by severe undernutrition (17, 283). It seems certain that body and tissue responses to insulin, epinephrine, atropine, pilocarpine, and histamine are greatly depressed in severely starved persons (50, 95, 111, 226). Thrombotic phenomena were remarkably frequent in extremely undernourished persons (50, 111, 175, 226).

TUBERCULOSIS

During and after World War II, one of the most serious health problems was tuberculosis; this was generally blamed on the nutritional state. In all areas of food shortage there was an increase in both morbidity and mortality from this disease which was roughly proportional to the severity of the food restriction (77, 94, 102, 371, 377, 381). In some regions, such as in Great Britain, the rise in tuberculosis may have been overestimated because of improved ascertainment (241) and in most regions the factors of overcrowding and overwork cannot be evaluated separately. The relatively small increase in tuberculosis in Japan (376) cannot properly be blamed on malnutrition.

The most deplorable conditions were found in the German concentration camps where extreme undernutrition was combined with incredible crowding and disregard of elementary sanitation. Among persons rescued from these camps the incidence of tuberculosis ranged from 25 to 60 per cent, and these were largely the primary pulmonary form of the disease (83, 193, 223, 226). The new evidence adds to the conviction that caloric undernutrition greatly favors the development of tuberculosis but the mechanism remains obscure. A diminished reactivity to the disease process may be suggested. The febrile response is small and there is also a failure to show positive reactions to Mantoux and Pirquet tests (50, 111).

The course of tuberculosis in the severely undernourished person is most unfavorable if the dietary condition remains bad (63) and anything short of an abundant diet is undesirable (198). Where a full diet is combined with good care the course of the disease in the emaciated tubercular patient may be very satisfactory (223).

NUTRITIONAL NEUROPATHIES IN GENERAL

Recognition of the importance and protean nature of nutritional neuropathies was greatly extended as a result of the experiences of World War II, primarily in Japanese prison camps. The general subject was examined in scholarly detail by Spillane (350) and by Denny-Brown (86), who provide extensive bibliographies with many recent papers. Throughout southeast Asia every prison and internment camp had its complement of cases of "burning feet," "camp eyes," and parasthesias in great variety. These conditions

were absent or extremely rare in most of Europe but some cases occurred among German troops in the Middle East and the Channel Islands as well as in a few men from prison camps in eastern Europe (350). Besides the more familiar peripheral neuritis of simple thiamine deficiency, other prominent syndromes related to malnutrition included: retrobulbar neuritis, spinal ataxia, spastic paraplegia, "burning feet," corneal changes, deafness, and a myasthenic bulbar syndrome (86).

The incidence of these neuropathies among the prisoners of the Japanese is variously reported. Among Canadian soldiers captured at Hong Kong some degree of partial optic atrophy was seen in 20 per cent (28). Among 2,500 civilians interned at Hong Kong there were 756 cases of "burning feet" and 370 cases of nutritional retrobulbar neuropathy (345). There were 409 cases of dim vision counted among rescued prisoners of war in Siam (158). A much lower incidence is suggested from a systematic screening of rescued personnel in the whole southern Asia area. Out of 60,000 released British and Indians, 3,667 were received in hospitals in India in September and October, 1945; careful study of the latter revealed 303 clear cases of neurological disorder (86). However, surveys on survivors certainly underestimate the incidence of nutritional neuropathy in the war in the Far East. Toward the end of 1942 about 60,000 men were sent by the Japanese to labor in Siam and roughly 80 per cent of them died in three years; there was little general starvation but deficiency of vitamins of the B complex was believed to have been a major factor in this extreme mortality (98). One must assume a high incidence of neuropathy in the 48,000 men who died.

NUTRITIONAL RETROBULBAR NEUROPATHY

With regard to incidence, disability, and persistence, probably the most important of the neuropathies seen in World War II is retrobulbar neuritis, popularly known as "camp eyes" among the Dutch victims (85). This was not confined to inmates of the Japanese camps and prisons. At the free dispensary of the Philippine General Hospital, 467 cases were seen in 20 months (108). Moreover, this condition is not confined to Asia; a study of colored children in Jamaica disclosed 74 cases of an identical or very similar disorder (391). But wherever seen, the clinical features were similar, with dim and blurred central vision, without diminished peripheral

fields and usually without disturbed night vision (86, 176, 283, 306, 348, 350). More detailed examination revealed relative or absolute central or paracentral scotomata, and sometimes small retinal hemorrhages (66, 85, 86, 329, 331, 350). Blurring of the vision was the first sign and this was often followed by burning, photophobia, lachrymation and retrobulbar pain (43, 176). In well-established cases, 70 to 80 per cent showed changes in the fundus (85).

While there is general agreement that deficiency of the B vitamins, notably thiamine, is involved in nutritional retrobulbar neuritis, it has been observed repeatedly that there is no necessary association with beriberi or pellagra (85, 306). In the Netherlands East Indies "camp eyes" occurred in camps where beriberi was practically nonexistent and in persons who displayed normal thiamine excretion and normal blood pyruvic acid (283). A toxic factor, possibly an antivitamin, has been suggested (28, 66). Early treatment with yeast, B vitamin mixtures, thiamine, or simply a good diet is reported to have been successful (85, 158, 331, 391), but at Singapore there was no success with yeast, thiamine, niacin, liver extract, rice polishings, and a good diet (141, 388); the Singapore patients had a general encephalopathy and "burning feet" as well as changes in vision. There is full agreement that the condition is irreversible in long-standing cases (85, 158, 350). Intensive dietary treatment of six patients for several years had no effect (310). In the Canadians recovered from Hong Kong, the loss of visual acuity was the most persistent of the numerous defects of nutritional origin (72).

THE BURNING FEET SYNDROME

"Burning feet" (lightning foot, electric foot, causalgia, ignipedites, nutritional melalgia) was the subject of five reports more than 100 years ago and at least 19 papers on this condition appeared before World War II (129, 350), but it had received little attention from nutritionists until it affected thousands of men in Japanese prison camps (72, 86, 101, 194, 293, 350, 358, 393). The burning disesthesia in the feet, sometimes also in the hands, was prominent in regions where "camp eyes" occurred but was apparently independent of the neurological changes observed in beriberi neuritis (132, 350), though the two conditions sometimes occurred together (72, 132, 393). Among American soldiers imprisoned in the Philippines the condition was epidemic by the end of 1942 and the clinical picture is fully described (129, 194).

D. A. Smith (345) who reported 756 cases, considered thiamine was not involved. Treatment with thiamine and niacin was ineffective in the Philippines (129); Gopalan (132) tried riboflavin as well as thiamine and niacin without effect but had good results with yeast and with calcium pantothenate, the latter in daily injections of 40 mg. The possibility of a toxic factor has been entertained and abandoned (283).

Gopalan (132) considered there was a primary vasomotor fault, as in erythromelalgia, but a direct nerve lesion cannot be excluded (350). Professor Kinoshita of Japan stated that necropsies revealed diffuse thickening in the walls of the small arteries but no changes in the cord or peripheral nerves (129).

NUTRITIONAL ENCEPHALOPATHY

The occurrence of numerous cases of what seemed to be Wernicke's encephalopathy among prisoners of war at Singapore (141, 388) adds new support to the belief that this condition is, or may be in some cases, of nutritional origin. Spillane (350) saw eight cases among malnourished prisoners of war as well as two others where there was no indication of malnutrition. Intensive treatment with thiamine is highly successful in some instances; at Singapore of 37 patients who had full treatment with thiamine, there were 11 deaths, one partial recovery, and 25 complete recoveries. The fact that 79 of the patients at Singapore had classical beriberi and that 4 of Spillane's 10 patients had peripheral neuritis, suggests agreement with de Wardener and Lennox's conclusion that this syndrome is a cerebral form of beriberi, but Spillane pointed out that something more was involved than a simple thiamine deficiency and Graves (141) could find no basis for relating the condition directly to athiaminosis.

MALNUTRITION AND SEX

Marked sexual differences in mortality under famine conditions were observed at practically all ages in Holland, females being much more resistant than males (22, 93, 94). Male children were thought to be more prone than females to famine edema in Greece (292); the same tendency has been reported in adults (161). Semi-starvation greatly reduces libido (119, 283, 289). In civilian practice in the United States nutritional deficiency was discovered in 143 patients with complaints of impaired libido, potency, and nympho-

mania; improvement from diet alone was claimed in 62 out of 76 individuals (40).

Gynecomastia in men suffering from severe malnutrition was observed in several Japanese prison camps. In the Netherlands East Indies many men and boys were affected during internment or thereafter (283). Among the American soldiers at Cabanatuan prison camp it was estimated that at least 10 per cent developed gynecomastia in camp and nearly 50 per cent had some degree of the disorder on return to the United States (181). Hibbs (166) saw some 500 cases, most of which developed when the diet was slightly improved after a long period of semistarvation and vitamin deficiency. These cases involved disc-like tumors most of which spontaneously regressed with a continued bad diet; in a few cases operation revealed what looked like fibroadenomata (72). In contrast, biopsies in another series revealed only proliferative changes (297). On return to the United States, 48 cases were studied in one series; in 17 of these men the gynecomastia first appeared when the men returned to a good and abundant diet (214). S. S. Platt *et al.* (299) report, without details, similar hypertrophy of the breast in men recovering from malnutrition caused by diarrhea and in one obese patient who was on a reducing diet and then went on a food orgy. Failure of the liver to inactivate estrogens in these patients is suggested (214, 299). Studies on hormones in the urine were not revealing (317).

DENTAL CARIES

The most common physical defect in the United States and in many other parts of the western world is dental caries. For many years it has been believed that nutrition exercises a dominant role in the development of tooth decay and the high incidence of caries has been widely cited in pious exhortations about calcium, phosphorus, vitamin D, and the "basic seven" foods. Recent evidence gives conclusive support to the concept that diet is of major importance, but it indicates also that the concept of a "good" diet with regard to caries must be revised. Actually, there seem to be only two nutritional conditions associated with a "low" incidence of dental caries: (a) a moderately high intake of fluorides, and (b) a substantial food shortage.

The story of fluorides and caries cannot be discussed here, but the evidence from areas of food shortages is equally dramatic.

During World War II there were variable degrees of general undernutrition, without specific vitamin deficiencies, in all of Europe; in the Japanese prison camps general undernutrition was coupled with multiple vitamin deficiencies. In none of these areas was there an increase in the incidence of caries; most of the data show a substantial decrease. In Norway (370), in Switzerland (110), and in England (256, 287), surveys of children revealed definite but moderate decreases in the incidence and severity of dental caries. Attempts to explain these changes have stressed "improved" nutrition and have pointed to the food program in England which involved milk distribution to children and added calcium in bread (211, 256), while disregarding the fact that the "improved" diet contained less fruit, protein foods, and calories, and more phytic acid. Moreover, an equally low or lower incidence of caries was observed where nutritionists did not exert their beneficial influence on food distribution or bread fortification.

The very ill-fed children of the Channel Islands had fewer caries than the children in England (217). In four years of war there was a 50 per cent reduction in the incidence of caries in the abandoned children of Paris collected at the center of Saint Vincent de Paul (84). The relatively malnourished children in Italy, who were unaccustomed to oral hygiene, had less than one fourth of the many dental defects found in the far "better" fed children of the United States (325).

The improvement in caries in the areas of food shortages was not limited to the children. There was a sharp decrease in dental caries in adults in urban Holland (355). Adult Italians subsisting on "bad" diets had far fewer carious lesions than American adults (325). Even in the most extremely malnourished persons in the prison camps, incidence of dental caries was lessened (84). Among persons repatriated after long years of severe malnutrition in Japanese camps "the number of carious teeth was less than that of the same age group living under normal conditions" (276).

In the United States there is also accumulating evidence that persons who are judged definitely malnourished in other respects have far fewer dental defects than well-nourished controls (244). An interesting experiment on 97 children in varying nutritional states showed that dental defects initially were twice as numerous in the well-nourished as in the malnourished children and that 12 months of providing a quart of milk daily to 25 of the latter was

associated with an incidence of new caries amounting to 34 per cent of the initial defects while the 47 malnourished children who received no dietary "improvement" had a corresponding figure of 21 per cent (96).

It may be suggested that findings such as those cited above indicate an advantage in coarse and cheap foods, with little free sugar (359). The role of caloric restriction per se cannot be evaluated as yet. But in any case it is clear that previous definitions of "good" diets must be drastically revised if the diets are to be evaluated in terms of incidence of dental caries. The role of the diet must be considered with respect to the bacteria in the mouth, the character of the saliva, and the complex of factors involved in the composition of the tooth itself. The relation of diet to the latter continues to be studied in animals (18, 347). Animal studies with penicillin indicate the importance of the oral flora (252, 405). The importance of fermentable carbohydrates (10) and of the physical nature of the diet (11) are again emphasized in animal studies. Speculation on the value of "tough" food is interesting (284).

HEMOGLOBIN AND IRON NUTRITION

The hemoglobin level in various population groups continues to be a favorite subject for surveys though there is no clear agreement as to the significance of the data gathered (82, 105, 157, 229, 295). Experiments with 800 Indian Army recruits suggest the value of extra iron as well as better rations in reducing the high incidence of anemia (179). Calculations from studies on children with radioactive iron indicate the daily requirement for absorbable iron is small (80). Iron absorption by women on different diets has been studied (185).

Darby (78) has reported six cases of angular stomatitis and superficial glossitis with mild chronic anemia. Some of these responded to iron therapy alone, others were successfully treated with iron after the failure of prolonged and intensive treatment with various preparations of B vitamins. This experience resembles that of Marcussen (246), who treated 100 cases of cheilosis and angular stomatitis with riboflavin without success but in five of the patients who had sideropenia all abnormality quickly responded to iron. A controlled experiment in an antenatal clinic in London indicated that the routine administration of iron reduced the so-called physiological anemia of pregnancy without, however, any

effect on the incidence of toxemia, puerperal sepsis, or infant mortality; there may have been some effect on postpartum hemorrhage (399).

All reports agree that caloric undernutrition results in a moderate anemia which is independent of iron intake. Lehmann (231) concluded that a low level of hemoglobin is a more reliable index of malnutrition than a low value for serum protein. The low blood iron levels in infection are not corrected by iron administration (142).

SCURVY AND ASCORBIC ACID DEFICIENCY

As an example of the present latitude for controversy about the "required" or "recommended" intake for man of a specific nutrient, the case of ascorbic acid is as suitable as any. The beginnings of an analysis of intra-individual variation with regard to this vitamin are seen in the data for the relation between intake and the plasma level in a homogeneous group (41 young women); for the several levels of intake the standard deviation for the plasma concentration was of the order of ± 20 per cent of the mean over short periods (91). Individual renal thresholds for ascorbic acid may be constant from year to year (216).

Dogramachi (92) reported seeing 241 "typical" scorbutic infants in 10 years; in most cases the mothers were aware of the need for vitamin C and a number insisted that their children had orange juice regularly, but no analysis of intake was provided. Experimental studies on the relation between test dose intake and the level of ascorbic acid in blood and urine continue to be interpreted as indicating high requirements (151, 360), which may be contrasted with some data on ascorbic acid intake. In Toronto in late winter, 16 to 20 per cent of a surveyed group received less than 5 mg. daily, 38 per cent of the children had less than 20 mg., and 82 per cent of the adults had less than 30 mg. of ascorbic acid daily (311); nothing whatever was said about signs or symptoms in these people. Among Bantu soldiers the average actual intake of ascorbic acid was only 15 mg. daily but signs of scurvy were rare and the mean plasma level in 361 men was 0.58 mg. (200). In 15 representative maternity hospitals in Great Britain the mean diet provided 31.2 mg. of ascorbic acid (74). A careful field trial with 2,500 soldiers in Northern Sweden indicated that three months of dosing with ascorbic acid produced large changes in blood and urine levels

but none in performance or any of the numerous aspects of health evaluated (76).

There are several instructive reports on the cure of scurvy with small doses of ascorbic acid given under controlled conditions. A 55-year-old man with typical severe scurvy was hospitalized and completely cured in seven weeks on a diet of nothing but sterilized milk plus 20 mg. of ascorbic acid daily (23). A 42-year-old woman admitted to hospital with the full picture of scurvy was put on a diet free of ascorbic acid and given 15 mg. of the vitamin daily (243). Improvement began in three days and she was substantially normal in four weeks at which time she was put on a regimen of 150 mg. of ascorbic acid; the larger dosage had no particular effect. In both of these cases the previous diet accounted for the scurvy. At Sheffield, seven men subsisted on a diet with 10 mg. of ascorbic acid daily and 10 men on only 1 mg. (68). No signs of deficiency appeared in the former group in 14 months. First signs of deficiency appeared in the latter group after 190 to 240 days and frank scurvy was evident one to two months later. A dosage level of 10 mg. of ascorbic acid daily produced fairly rapid improvement and doubling this had little effect. It was agreed that 30 mg. of ascorbic acid daily is adequate for adults. Sinclair (341) notes the complexities of interpreting data on blood ascorbic acid.

J. D. King (212) has reviewed the general problem of the relation of the diet to paradontal disease; it is clear that ascorbic acid is only one of many dietary items involved and nondietary factors are numerous and important. The use of capillary strength tests in the diagnosis of vitamin deficiency is critically reviewed; a large minority of scurvy cases do not show positive capillary strength changes and most studies fail to show a relation between capillary fragility and blood ascorbic acid on the results of tolerance tests (279).

BREAD AND ABSORPTION OF CALCIUM AND NITROGEN

The widespread shift to high extraction flour and dark breads in World War II (97) resulted in concern about the absorption of nutrients, particularly calcium and nitrogen, from such materials. Very fine milling of wheat bran has been advised to promote digestion (37), but this measure was useless in rat studies (288). Nitrogen balance studies on normal people subsisting for short periods on diets with large amounts of various breads have produced rather

inconclusive results. Trémolières and his colleagues (149, 373) concluded that nitrogen absorption is so bad from breads made with 98 to 100 per cent extraction flour that these lose their presumed advantage, but other studies in France indicated only a doubtful handicap for 98 per cent extraction flour (305). In England, various complications were recognized but it was concluded that all of the protein contained in breads made with 80 to 90 per cent extraction flour is completely digested (250).

The addition of calcium carbonate to bread is reported to increase calcium retention (73). The greatest concern has been over the effect of phytic acid on calcium balance but the situation in human nutrition is not yet settled. There is no doubt that the sudden addition of large amounts of phytate to the diet produces an immediate reduction in calcium absorption and the balance may become negative (169, 170). The formation and solubility of calcium phytate is much affected by the dough formula and the bread making procedure (271, 327). The Danish Cereals Committee (61) estimated that 25 to 30 per cent of the daily calcium intake of Copenhagen workers was unavailable in 1946 because of the action of phytic acid and recommended baking procedures which would destroy phytic acid.

With this problem, as well as in many others in nutrition, it may be that proper consideration of adaptations in the human body would lead to different conclusions. Walker, Fox & Irving (384) made careful studies on four men for 7 to 19 consecutive weeks and controlled conditions to provide critical information on the relation between phytate intake and calcium balance. With high phytate (standard "war bread") there was an immediate reduction in calcium balance but retention improved as the diet was continued and eventually the losses were made good. This excellent study justifies the conclusion that whole cereal diets with much phytic acid are not necessarily harmful to calcium balance. Similar possibilities of adaptation must be allowed for in considering the report that calcium utilization from soybeans is very low (326).

NUTRITIONAL FACTORS AND INTELLIGENCE

Hope that simple dietary measures may aid the mentally deficient, or even improve the intelligence of ordinary people, continues to be reflected in some research studies with glutamic

acid and with thiamine. The initial choice of these nutrients came, at least indirectly, from *in vitro* demonstrations that they have special metabolic effects in brain tissue; the work of R. A. Peters on thiamine in the brain is now well established and glutamic acid seems to be the only amino acid metabolized by brain slices.

Tests of earlier claims for a beneficial effect of glutamic acid on learning in rats have been inconclusive (153) and in more elaborate studies, completely negative (249, 357). However, reports of favorable results on patients continue to appear. Zimmerman *et al.* (406) administered glutamic acid to 69 patients with convulsive disorders but with widely varying intelligence; analysis of the effects of a year's treatment with 30 patients indicated an average gain of about 11 I.Q. points in the first six months with little change thereafter. Albert, Hoch & Waelsch (5) reported striking improvements in a few months of glutamic acid (about 10 gm. daily by mouth) treatment of eight mentally defective persons; "controls" with placebos were used. Though a metabolic disorder of the brain that may be corrected with glutamic acid could be suggested for the feeble minded patients, the reported favorable effect on persons of good intelligence (406) is more puzzling. Independent new researches are needed.

The claims by Harrell for pronounced benefits from supplementary thiamine given to children in an orphanage have emphasized improvement in items of intelligence (154). The full presentation of the results is a trifle less exuberant than earlier reports but insists that the addition of 2 mg. of thiamine daily to a fair diet may "spell the difference between alert and successful living and a marginal effectiveness" (155). A critical study of the effects of added thiamine on items similar to those studied by Harrell has been made on 44 pairs of identical twins (312a). After pretest studies, supplementation continued for 136 days (36 pairs) and 273 days (25 pairs). The final results showed no effect whatever, although half of the subjects were getting from 20 to 40 per cent less than the National Research Council-recommended intake of thiamine. Attempts to demonstrate improved learning from added thiamine in rats have also been negative (248).

In the Minnesota Experiment elaborate tests failed to reveal any deterioration of mental ability resulting from severe caloric undernutrition but spontaneous mental activity declined (119); basic intelligence seems to be much more resistant to change than is intellectual behavior.

FAT METABOLISM AND OBESITY

For some time it has been apparent that much more information is needed on the relation between the fat level in the diet and the caloric balance. Two laboratories have been experimenting with rats on this question but studies on other species, particularly man, are wanted (113 to 116, 321, 322). In 41 normal persons who received 50 gm. of fat daily, the fecal fat averaged 5 per cent of the intake; data are also presented for 120 patients with steatorrhea (70). Walker (383) in a prolonged and careful study on four men, found that the fecal fat was from 5 to 6 per cent of the intake on intake levels of 55 to 115 gm. of fat daily; this was unaffected by whole grain bread. In short-period studies on 40 students there was no effect of fat level or type (animal or vegetable) on the amount of fecal fat, fecal nitrogen or gastrointestinal symptoms; the authors questioned the meaning of "fat balances" (14, 15). A surface active agent (polyoxyethylenesorbitan mononucleate) is reported to increase the absorption of fats and fat soluble substances (187). The fecal fat averaged 4.0 per cent of an intake of 102 gm. of fat daily and 4.2 per cent on an intake of 208 gm. of fat daily; the high fat diet was very slightly inefficient in caloric absorption but it did not affect nitrogen loss (402). In 29 patients with idiopathic steatorrhea, fecal fat averaged 27 per cent; in 21 patients with tropical sprue the average ranged from 7 to 37 per cent according to the stage of the disease (70). The etiology and metabolism in steatorrhea has been discussed by Frazer (120, 121).

Attempted subsistence on pemmican produced nausea in a day or two but the high fat level was not itself considered really troublesome (69). Prolonged (36 months) administration of daily capsules containing 100,000 I.U. of vitamin A and 8.5 mg. of cholesterol produced a moderate progressive rise in the blood of both vitamin A and cholesterol (378a). In 1,250 necropsies on persons 35 years of age and older the incidence of general atherosclerosis was directly proportional to the amount of adipose tissue in the body (396, 397). It is suggested that a high fat diet may produce atherosclerosis in man (273). Recent evidence indicates that in severe undernutrition the concentration of cholesterol in the blood is from slightly to markedly subnormal (47, 146, 375).

Recent papers on obesity contribute little beyond agreement with the sentiment expressed by Gastineau & Rynearson (127)

that "obesity is one of the most pressing and dangerous health problems we face today" (107, 180, 374). Skepticism about the views of the "caloric school," contributes nothing else (131). From 307 courses of therapy with 132 markedly obese patients it is concluded that anorexigenic aminopropanes are useful under strict medical control (398).

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METABOLIC INHIBITORS¹

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This review has been limited to a consideration of those metabolic inhibitors which exert their effects by virtue of their structural similarity to compounds playing a role in metabolism and growth. Previous reviews by Woolley, by Welch, and by Roblin (1 to 6) have shown the concept of competitive antagonism toward essential metabolites by compounds of similar structure to be so well established as to require little further justification.

General considerations.—The high hopes held forth for the development of new chemotherapeutic agents based on a rational application of this principle [e.g. (7)] have not yet been realized. However, such metabolic antagonists have already begun to be of service in elucidating metabolic functions of certain metabolites, as well as the pathways of certain biosyntheses.

A technique termed "inhibition analysis" has been applied in a series of papers by Shive and his co-workers to the study of the effect of a number of antagonists on bacterial growth (8 to 16). The procedure is based on the assumption that, if a metabolic inhibitor blocks a particular reaction and thus limits bacterial growth, addition of the product of the blocked reaction should permit growth. At higher concentrations of the antagonist, a second reaction may be inhibited, whereupon the addition of the product of the second reaction then still further reduces the sensitivity of the organism to the inhibitor. A number of agents other than the competing metabolite itself may reverse the inhibiting effect of a metabolic antagonist, each giving characteristic changes in the inhibition index and showing characteristic competitive or noncompetitive behavior (16).

That too facile interpretation of the results of inhibition analysis is possible was pointed out by Hitchings *et al.* (17), when it was observed that an entirely abnormal compound, 5-bromouracil, was implicated as a metabolic product of a reaction in the growth of *Escherichia coli* inhibited by 5-nitrouracil. Other difficulties in

¹ This review covers the period from January, 1947 to December, 1948.

interpreting numeral metabolite inhibitor ratios have been indicated (18).

The basic postulate for competitive antagonists is that they, by virtue of structural similarities, reversibly combine with an enzyme or other system at the same point as the essential metabolite with which they compete. However, Bartlett (19) has observed that the oxidation of D-alanine by D-amino acid oxidase was inhibited by benzoic acid and certain derivatives in a strictly competitive manner. The structures of glycine and benzoic acid are sufficiently dissimilar to make such competition puzzling. One possible suggestion for the competition of structurally dissimilar molecules was developed by Pfeiffer (20) for the special case of the muscarinic drugs in which certain chemical "prosthetic groups" were arranged at distances approximating their position in acetylcholine even though the intervening structure was vastly different.

p-Aminobenzoic acid (PAB) antagonists.—Considerable advance has been made in defining the mechanism of growth inhibition brought about by the sulfonamides since the subject was last reviewed (6, 21, 22). Recent experiments have continued to indicate that one of the major effects of the sulfonamides on microorganisms involves the blocking of the incorporation of PAB into pteroylglutamic acid (23 to 28). It has been suggested that the sulfonamides interfere with pteroylglutamic acid syntheses at the stage of the combination of PAB or *p*-aminobenzoyl glutamate with pteridinaldehyde (29).

The participation of PAB in the synthesis of methionine is likely since methionine can overcome the inhibition of the growth of *E. coli* by sulfonamides (9, 30). One PAB antagonist, 2-chloro-4-aminobenzoic acid, has been shown to be a very specific inhibitor of the methionine synthesizing system of *E. coli*, but to have little or no effect on other systems involving the participation of PAB (9, 31).

Another function for PAB that has been clearly established is its role in the synthesis of the purine bases. Thus, the inhibition of growth of *E. coli* and other bacteria by the sulfonamides can be counteracted by xanthine, guanine, and other purines providing methionine or a small amount of PAB is present in the medium (9, 32). A diazotizable amine was isolated by Stetten & Fox (33) from cultures of *E. coli* inhibited with sulfadiazine and has been

identified by Shive *et al.* (34) as 5(4)-amino-4(5)-imidazolecarboxamide. Presumably, PAB or a derivative functions in the further conversion of this imidazole into a purine by participating in the introduction of a carbon atom to complete the pyrimidine ring. Based on the observation that glycine stimulates the synthesis of the above intermediate by *E. coli* inhibited with sulfadiazine, it has been suggested that glycine is a precursor of this intermediate and therefore of purines (35).

PAB also appears to be involved in the metabolism of asparagine, cystine, and tryptophane by *Salmonella paratyphi*, since these substances act to overcome inhibition by sulfonamides (36).

It has been possible in *E. coli* successively to inhibit with sulfonamides a number of enzyme systems involving the participation of PAB. In *E. coli* the step most sensitive to the action of the sulfonamides is the synthesis of methionine. This is followed by reactions involving the production of xanthine and other purines (9, 30). The syntheses of serine and of pteroylglutamic acid are inhibited by the sulfonamides only at still higher concentrations.

Previous reviews have indicated that the bacteriostatic activity of the sulfonamides may depend upon their acid dissociation constants or upon their resonance forms. The *p*-aminophenyldimethylsulfonium salt of β -naphthalenesulfonate, which is capable of resonance, shows a low order of antibacterial action against *Staphylococcus aureus*, the inhibition being reduced by PAB (37). The phosphonous acid analogue of PAB has been prepared and found to inhibit growth of *E. coli*, the inhibition being reversed by PAB (38). *p*-Aminophenylsulfamide has also been shown to be a PAB antagonist with *Aspergillus* (39).

An interesting case of reversal of the usual relationships of PAB and sulfonamides has been described by Emerson (40, 41) in two mutants of *Neurospora* which require sulfonamide for growth. A double mutant of *Neurospora* requires both sulfanilamide and PAB for growth, a ratio of 1000:1 giving maximum growth, lower growth resulting at higher or at lower ratios. The requirement for sulfanilamide of the sulfur-requiring mutant has been shown by Zalokar (42) to be due to an overproduction of PAB by the mutant. The double mutant was found to be very sensitive to PAB and thus to require sulfanilamide as a detoxicant (42).

Pteroylglutamic acid (PGA) antagonists.—A number of compounds structurally related to PGA have now been shown to be

competitive antagonists of PGA. A "7-methyl folic acid" containing D-glutamic acid prepared by Martin *et al.* (43) inhibited the growth of *Streptococcus faecalis* R in a competitive manner, the inhibition being overcome by PGA. An analogous compound containing L-glutamic acid prepared by Franklin *et al.* is also an active PGA antagonist for *S. faecalis* R (44), the reversing effect of PGA depending on the presence of thymine and adenine (45). *Lactobacillus casei* (44), rats (44), mice (46), chicks (46), and swine (47, 48) were also inhibited by this antagonist.

Hutchings *et al.* (49) prepared pteroylaspartic acid substituting aspartic acid for the glutamic acid of PGA, yielding a competitive PGA antagonist for *L. casei*, *S. faecalis* R, and the chick, but lacking antagonistic action in *E. coli* and the rat (49).

It is interesting that combination of a methyl group in the pteridine nucleus and substitution of aspartic for glutamic acid produced a PGA antagonist (7-methylpteroylaspartic acid) having the same inhibition index for *S. faecalis* R as the aspartic acid substitution alone (50).

Substitution of a benzimidazole for the pterin nucleus of PGA was shown by Edwards *et al.* (51) to yield a derivative with appreciable PGA activity for *S. faecalis* R. Further substitution of a sulfonyl group for the carboxyl group of the *p*-aminobenzoic acid portion yielded a PGA antagonist.

Substitution of an amino group for the 4-hydroxyl of PGA produces a very active antagonist, 4-aminopteroylglutamic acid, which inhibits the growth of *S. faecalis* R (52, 53), and also produces symptoms of PGA deficiency in chicks (53), mice (54), rats (53, 55, 56), guinea pigs (57), and man (58). The toxic effects in mice, rats, and chickens are not appreciably reversed by PGA, and even in *S. faecalis* R the reversal is not strictly competitive.

A series of N¹⁰-alkyl PGA and pteric acids, including 4-amino-N¹⁰-methyl PGA, have also been shown to be PGA antagonists for *S. faecalis* R. (56, 59). Little reversal of the toxic action of the 4-amino compound was apparent in rats (56).

A series of 2,4-diaminopterins have been shown by Daniel and co-workers (60, 202) to exert anti-PGA activity in a number of microorganisms. 2-Amino-4-hydroxy-6,7-dimethylpteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine inhibited growth and hemoglobin formation in chicks, the effect being counteracted by PGA (61).

The compounds 2-amino-4,7-dihydroxypteridine-6-carbonyl-*p*-aminobenzoylglutamic acid and quinoxaline-2-carboxyl-*p*-aminobenzoylglutamic acid have been shown by Woolley & Pringle (62) to exert anti-PGA activity in *L. casei*.

Norris & Majnarich have studied the inhibition of cell proliferation of normal cells in tissue culture by 2-amino-4-hydroxy-7-methylpteridine and by xanthopterin-7-carboxylic acid, the inhibition being relieved by the addition of xanthopterin (63, 64, 65). The above situation appears to be reversed in tissue cultures of Brown-Pierce carcinoma, the growth being inhibited by xanthopterin and the inhibition being relieved by the above antixanthopterins (66). The replacement of two nitrogen atoms of xanthopterin by carbon to form quinoxaline has been reported by Hall (67) to yield a competitive antagonist to PGA for the growth of *S. lactis* R.

Evidence has accumulated suggesting that the PGA antagonists interfere with the synthesis of purines and pyrimidines. Thus, Stokstad *et al.* (68) have indicated that growth of *S. faecalis* R is inhibited by methylfolic acid in the presence of either adenine or thymine alone, but not in the presence of both. Thymine was ineffective in producing growth or hematologic responses in PGA-deficient rats or chicks and did not protect rats against methylfolic acid.

Application of inhibition analysis to the inhibition of growth of *L. casei* by a methylfolic acid in the presence of PGA, purines, thymine, and their combinations suggest that PGA functions in the biosyntheses of purines and thymine or their equivalents (15).

The possibility that PGA or a derivative may act in a coenzyme system was raised by the observation of Martin & Beiler (69, 70) that 7-methylfolic acid and pteroylaspartic acid inhibit the activity of dopa decarboxylase from rat kidney, the inhibition of the former being overcome by PGA. A hypotensive action in the dog observed with 7-methylfolic acid (71) might be explained on the basis of the inhibition of tyrosine decarboxylase. However, tyrosine decarboxylase of kidney tissue was inhibited only at high concentrations of methylfolic acid (69).

The suggestion that PAB or a derivative is involved in the biological transfer of single carbon units (34) gains some support from the observation that formylfolic acid is more active in overcoming the inhibition of *S. faecalis* R by 7-methylfolic acid than is PGA itself (72).

The pronounced inhibiting action of PGA and pterioic acid on the oxidation of xanthopterin or xanthin by their respective oxidase systems from liver or milk (73) appears to be due to 6-pteridyl aldehyde (74).

The suggestion that PGA might function as a metabolic antagonist for glutamic acid (75) has not been substantiated (76).

Methylfolic acids and 4-amino-PGA cause an increase in the erythrophagocytic activity of granulocytes in bone marrow cultures (77) raising the possibility that some of the anemias associated with folic acid deficiency may be associated with increased erythrophagocytosis.

Pteroyltriglutamic acid has been observed to exert a pronounced inhibiting effect on the growth of Sarcoma 180 transplants in mice, while PGA itself did not (78, 79). Studies on the effect of pteroyltriglutamic acid on human neoplastic disease have been reported (80, 81), the results being equivocal. The growth of Rous sarcoma in chickens was prevented by PGA deficiencies as well as by administration of antagonists of PGA (82).

Purine and pyrimidine antagonists.—A number of thymine antagonists have been prepared by Hitchings and associates (83, 17), some of which were inhibitory to the growth of *L. casei*. Principal among these were compounds in which the methyl group of thymine was replaced by halide, amino, hydroxyl, or nitro groups to form the 5-substituted uracils. These inhibited the growth of *L. casei*, the inhibition usually being overcome by thymine or by PGA. The inhibition by 5-bromouracil, however, was antagonized by PGA, but not by thymine, suggesting that PGA cannot function solely by participating in the synthesis of thymine. The addition of 5-bromouracil reduced the toxicity of 5-nitrouracil to *L. casei*. The 5-chlorouracil and 5-bromouracil nucleosides containing the pyranose forms of D-ribose, D-arabinose, D-glucose, and D-galactose have been synthesized (84) and reported to be biologically inactive on wild types and uracil-requiring mutants of *E. coli* and *Neurospora*, as well as *L. casei* or *S. faecalis* R (85, 86).

Strandskov & Wyss (87) have shown that thiouracil prevents growth of *E. coli* and *L. casei*, a protective action being exerted by uracil. Thiiothymine also inhibits *L. casei* with thymine reversing the toxicity. No inhibition by these thiopyrimidines was observed in the presence of PGA. Thiouracil has also been observed by Wolff & Karlin (88) to have antiuracil action in *L. casei*. How-

ever, nucleic acid, nucleotides, or nucleosides did not reverse the thiouracil effect, suggesting that thiouracil probably was not effective by virtue of blocking incorporation of uracil into nucleic acid (89). It has been suggested that the goiterogenic effect of the thiopyrimidines in higher animals may be due in part to their antagonistic action toward the corresponding naturally-occurring pyrimidine (90). The only experimental evidence (91) for this hypothesis, however, is not strong.

Substitution of a nitrogen for the carbon of the imidazole ring of adenine and guanine to form triazalopyrimidines (92) and the substitution of carbon for nitrogen in the pyrimidine ring to form benzimidazole (93) has produced potent purine antagonists. The inhibition of growth of *E. coli* by benzimidazole was overcome by ribose nucleic acid but not by equivalent amounts of adenine in experiments of Klotz & Mellody (94). 2-Aminopurine inhibits growth of *L. casei*, the inhibition being overcome by thymine, adenine, or folic acid (95).

Nearly all 2,4-diaminopyrimidines, most 2-aminopyrimidines, and several 2-amino-4-hydroxypyrimidines were antagonists of PGA and of purines and of pyrimidines in *L. casei*. The inhibiting activity of 2,6-diaminopurine on *Lactobacilli* is relieved by adenine and by PGA (96).

Biotin antagonists.—A considerable number of biotin analogues have proved to be competitive antagonists of biotin (4, 6). One of the most studied of these has been desthiobiotin. This compound is utilized by yeast and a number of other biotin-requiring organisms apparently by being converted into biotin (97). Desthiobiotin, however, is a competitive antagonist of biotin in the growth of *L. casei* (98, 99), the activity being restricted to the isomer corresponding to the naturally-occurring *d*-biotin (100).

Removal of the methyl group of desthiobiotin yields 2-oxo-4-imidazolidonecaproic acid, which is a biotin antagonist for *L. casei*, for yeast, and for *E. coli* (14, 98, 99, 101). Substitution of an ethyl for the methyl group of desthiobiotin also produces an antibiotic (99). Imidazolidonecaproic acid blocks the conversion of desthiobiotin to biotin in *E. coli*, but not the further utilization of biotin. Desthiobiotin is thus an obligate intermediate in the synthesis of biotin by *E. coli* (14). Although desthiobiotin has biotin activity in *Saccharomyces cerevisiae*, the next longer aliphatic homologue (desthiomobiotin) is a biotin antagonist (102). Substitution of

longer or shorter side chains for the caproic acid moiety of imidazolidone aliphatic acids results in reduced antibiotin activity (101).

Biotin sulfone, although it is a biotin vitamer for yeast (103), has an antibiotin activity for *L. casei* (98). Substitution of a sulfonic acid for the carboxyl group of desthiobiotin creates an antibiotin agent for *S. cerevisiae* which is more effective against desthiobiotin or oxybiotin than against biotin (104). The sulfonic acid analogue of oxybiotin has also been prepared and found to have antibiotin and antioxybiotin activity (105, 106). It is of utmost interest that oxybiotin, a compound in which oxygen has been substituted for sulfur, exerts considerable biotin-like activity. This activity does not involve the conversion of the vitamer into biotin, but is an activity intrinsic in the oxybiotin molecule (107, 108).

Of a number of biotin homologues with longer or shorter fatty acid side chains, the one with one greater carbon (homobiotin) is a very potent antagonist of biotin in *S. cerevisiae* and *L. casei* (109). The corresponding sulfone is less potent an antibiotin in both species.

The fatty acid homologues of oxybiotin, especially homooxybiotin, act as potent antioxybiotins for *S. cerevisiae* and for *L. arabinosus*. They have, however, much weaker antibiotin activity (110).

A number of ureylenebenzene and urelenecyclohexane analogues of biotin have been prepared in which the five-membered, sulfur-containing ring was replaced either by benzene or by cyclohexane (111, 112). Many of these had antibiotin activities for *L. casei* or *S. cerevisiae*, the cyclohexane compounds being in general more potent antagonists.

Three biotin antagonists (2,3,4-urelenecyclohexylbutyric acid, homooxybiotin, and oxybiotin sulfonic acid) were shown by Axelrod, Purvis & Hofmann (106) to prevent the stimulating effect of biotin on the fermentation of biotin-deficient yeast. The stimulation of fermentation by aspartic acid in such biotin-deficient yeast was not prevented by the biotin antagonists, suggesting that biotin may be concerned with the formation of aspartic acid. That aspartic acid or its precursors may be involved in the function of biotin also follows from work by Shive & Rogers (113) in which the inhibition of growth of *L. arabinosus* by 2,3,4-urelenecyclohexylbutyric acid was reduced by aspartic acid or oxaloace-

tate. Similarly, the inhibition of growth of *E. coli* by 2-oxo-4-imidazolidonecaproic acid was relieved by α -ketoglutaric acid or by glutamine (113).

Nicotinic acid antagonists.—The antinicotinic acid activity of pyridine-3-sulfonic acid or its amide, of 3-acetylpyridine, and of 5-thiazole carboxamide has already been mentioned in previous reviews (4, 6).

The injection of 3-acetylpyridine causes maldevelopment and death in chick embryos (114), the effect being reversed by nicotinamide and, less effectively, by nicotinic acid and by tryptophane. Pyridine-3-sulfonic acid inhibits the growth of oral *L. acidophilus* (115).

Pantothenic acid antagonists.—A large number of pantothenic acid antagonists have been discussed in previous reviews (4, 6). A potent antipantothenic acid activity for a large number of lactic acid bacteria was shown by Drell & Dunn (116) to be exerted by ω -methyl pantothenic acid (having a methyl group substituted for a hydrogen on the terminal hydroxyl group). Pantoyltaurine was somewhat less effective in this series. A combination of the two inhibitory changes (ω -methyl plus the sulfonyl group) to give the hybrid ω -methyl-pantoyltaurine produced an antagonist which was less active than the ω -methyl substitution alone. The suggestion was advanced that the two analogues interfere with the metabolism of pantothenic acid at different loci (116). Phenylpantothenone blocks the incorporation of pantothenic acid into coenzyme A in pantothenic acid-deficient yeast (117).

Several pantothenic acid analogues including α - γ -dihydroxy- β , β -dimethyl-*N*-(2-(phenyl mercapto)-ethyl)-butyramide inhibited competitively the growth of *Trichomonus vaginalis in vitro* but had no protective action *in vivo* (118).

Pyridoxine antagonists.—The first antagonist against pyridoxine was a desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) which was found by Ott (119) to be a strong antipyridoxine agent in pyridoxine-deficient chicks. This antagonist also aggravated the acrodynia of pyridoxine-deficient rats (120). Desoxypyridoxine given to tryptophane-fed, pyridoxine-deficient rats caused an increased excretion of xanthurenic acid (121). The histological changes in tissues of chicks, monkeys, and dogs induced by desoxypyridoxine include an anemia and atrophy of spleen, lymph nodes, thymus gland, and the blood forming con-

stituents of the bone marrow (122). Antibody responses were impaired and a marked atrophy of lymphoid tissue in rats and mice resulted from the administration of desoxypyridoxine (123). In connection with the atrophy of the lymphatic tissue, it was found that desoxypyridoxine administration decreased the number of takes of a transplanted lymphosarcoma in mice (124). Marked upsets in the reproduction of female rats given desoxypyridoxine on pyridoxine-deficient diets have also been noted (125).

Methoxypyridoxine (2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine) is also an effective inhibitor of pyridoxine in the pyridoxine-deficient chick (126) but appears to show some pyridoxine-like activity in affecting the tryptophane metabolism of pyridoxine-deficient rats (121).

While desoxypyridoxine had no effect on tyrosine decarboxylase prepared from *S. faecalis* R, it became a potent inhibitor of this enzyme when phosphorylated (127). Desoxypyridoxine, 2-methyl-3-hydroxy-4-hydroxymethylpyridine, and 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine were found by Martin and co-workers (128) to be competitive pyridoxine antagonists in *S. cerevisiae*.

Riboflavin antagonists.—A number of riboflavin antagonists have been considered in previous reviews (4, 6). These include analogues produced by the substitution of chlorine atoms for methyl groups, by a shift in the position of a methyl group, by substitution of a phenazine nucleus, and by substitution of different sugars for the ribityl group.

Hellerman and his associates (129, 130) have found that the flavine enzyme D-amino acid oxidase is inhibited, not only by atabrine, but by quinine, plasmochin, etc., and that the effects of these compounds are also antagonized by flavine-adenine-dinucleotide. This would suggest that structural similarity of certain antimalarials to the flavine nucleus is not the sole basis of their antimalarial action.

Thiamine antagonists.—The potent effect of pyrit'hiamine as an antagonist of thiamine in microorganisms has already been discussed in previous reviews (4, 6). Pyrit'hiamine has been shown to inhibit competitively the growth of *Phycomyces blakesleanus* and *Ustilago violaceae* (131) and the oral *L. acidophilus* (115) but to have no effect on certain strains of *B. paratuberculosis* (132).

Arginine antagonists.—L-Canavanine from jackbeans was

shown by Horowitz & Srb (133) to be a powerful inhibitor of certain wild type *Neurospora* strains. The effects of this agent is relieved competitively by arginine and also less effectively by lysine, and in one case by methionine. Canavanine also inhibits the growth of a number of bacteria (134), arginine overcoming the effect competitively both in those strains which require arginine and in those which do not.

Aspartic acid antagonists.—Shive & Macon (8) have found that *dl-p*-hydroxyaspartic acid (the isomer which is converted to *dl*-tartaric acid on treatment with nitrous acid) inhibits the growth of *E. coli* and *Leukonostoc mesenteroides*, the effect being competitively prevented by aspartic acid. Mesodiaminosuccinic acid was also an anti-aspartic agent in *E. coli* (8). These antagonists of aspartic acid are related to aspartic in having in one case an amino and in the other case a hydroxyl group on the β -carbon. The effect of *dl-p*-hydroxyaspartic acid could be overcome by glutamic acid, suggesting a function of this compound as a precursor of aspartic acid by transamination. Pantothenic acid, β -alanine, and asparagine were effective protectors against hydroxyaspartic, suggesting that aspartic acid might be precursors of these compounds (8).

Cystic acid was found by Ravel & Shive (11) to be an effective antagonist of the growth of *E. coli*, the effect being competitively overcome by aspartic acid. The effect appears to involve blocking by cystic acid of the decarboxylation of aspartic acid to β -alanine in *E. coli*, but not in *L. mesenteroides*. An inhibition analysis of the above system suggests that pantothenic acid is involved in the condensation of acetate or pyruvate with oxalacetate to produce *cis*-aconitate (135).

Glutamic acid antagonists.—Methionine sulfoxide is an effective antagonist against glutamic acid in the metabolism of *L. arabinosus* and *L. casei* (136, 137, 138). The antimetabolite probably blocks the amidation of the gamma carboxyl group to form glutamine, since this compound overcomes the effect of the methionine sulfoxide at concentrations much lower than the glutamic acid required. In support of this hypothesis, Elliott & Gale (139) have shown that the activity of an enzyme system from *S. aureus* which converts glutamic acid to glutamine is also inhibited competitively by methionine sulfoxide. Borek & Waelsch (137) have shown that the configuration about the asymmetric carbon atom of methionine sulfoxide appears to be of greater significance in determining the

antiglutamic acid activity than is the asymmetry of the sulfur atom, although the two diastereoisomers derived from L-methionine have significantly different antiglutamic acid activities. The amidation of the γ -carboxyl group of glutamic acid with ethylamine or ethanolamine has yielded inhibitors of *S. aureus* which were reversed by glutamic acid but not by glutamine (140).

The substitution of aspartic acid for glutamic acid in more complex molecules has provided competitive antagonists for folic acid (49) and streptogenin (141, 142).

An interesting observation of Waelsch *et al.* (138) is that the S-benzyl analogue of methionine sulfoxide is as highly active an antagonist of glutamic acid in *L. arabinosus* and *L. casei* as is the methionine sulfoxide, whereas ethionine sulfoxide is inactive.

Methionine antagonists.—One of the first amino acid antagonists was ethionine, the ethyl analogue of methionine, which was shown to be toxic to rats on a low methionine diet (143). This compound is also an antimethionine agent for *E. coli* (92). A strong lipotropic activity of ethionine was observed in rats fed a low choline, low methionine diet (144).

Methoxinine, an analogue of methionine in which the sulfur has been replaced by oxygen was shown by Roblin *et al.* (92) to be a strong competitive antagonist of methionine in *E. coli* and *S. aureus*. At a level of 22 mg. per day, methoxinine exerts a lipotropic effect in adult rats on a high fat, low protein diet, and at higher levels caused weight loss and death which could be prevented in part by addition of an equal amount of methionine (145).

Dittmer and associates (146) prepared the unsaturated amino acids allylglycine, methallylglycine, and crotylglycine as potential cysteine antagonists—these compounds being inhibitory toward the growth of *S. cerevisiae*. This group has also shown that 2-amino-5-heptenoic acid, the vinylene analogue of methionine, inhibits the growth of one strain of *E. coli*, the effect being reversed by methionine (147).

Phenylalanine antagonists.—A number of compounds involving a substitution for the phenyl group of phenylalanine have been shown to be competitive antagonists for this metabolite. The first of these to be prepared was β -2-thienyl-DL-alanine which was shown to inhibit, in competition with phenylalanine, the growth

of a number of microorganisms (4, 6). The inhibitory action of β -2-thienylalanine is entirely confined to the L-isomer (148). It appears that a major effect of β -2-thienylalanine involves blocking of the conversion of phenylalanine to tyrosine (13). β -2-Thienylalanine inhibits the growth of the human tubercle bacilli in Long's synthetic medium (149) and the growth of vaccinia virus in chicken tissue grown *in vitro* (150), the effects being reversed by phenylalanine.

A furyl analogue of phenylalanine (β -2-furyl-DL-alanine) has been prepared by a new synthesis (151) and shown to inhibit the growth of *S. cerevisiae* and of *E. coli* (152). Phenylalanine was the most active amino acid in reversing this inhibition, although leucine, tryptophane, isoleucine, and methionine also were active.

The pyrrole analogue of phenylalanine produced by substitution of nitrogen for a vinylene group of phenylalanine to form β -2-pyrrole-DL-alanine also produces a growth inhibitor for *S. cerevisiae* and *E. coli* which can be counteracted by phenylalanine (153).

Not only have antagonists to phenylalanine been produced by substitution of thienyl, furyl, or pyrrole nuclei for the phenyl group, but also by substitutions in the phenyl group itself. Beerstecher & Shive (10) have shown that β -hydroxyphenylalanine inhibits the growth of *E. coli*, or *L. arabinosus*, and of *S. faecalis* R, the inhibition being competitively overcome by phenylalanine at low inhibitor levels.

Replacement of one of the hydrogens of phenylalanine or tyrosine by fluorine to form 3-fluoro-phenylalanine or 3-fluoro-tyrosine has been shown by Mitchell & Niemann (154) to yield powerful competitive phenylalanine or tyrosine antagonists for *Neurospora*.

Tryptophane antagonists.—A number of modifications of tryptophane producing tryptophane antagonists have been considered previously (4, 6). These include indoleacrylic acid, naphthylacrylic acid, styrylacetic acid, and 5-methyl tryptophane. Indoleacrylic acids inhibits the synthesis of tryptophane by certain strains of *E. coli* and *B. typhosum* by blocking the coupling of serine and indole (155).

Some of the methyl indoles and methyl tryptophanes were found by Fildes & Rydon (18) to be very powerful antagonists of indole and tryptophane in their effect on the growth of *B. typhosum*.

The active methyl indoles appear to inhibit the coupling of indole with serine, while the methyl tryptophanes inhibit the further utilization of tryptophane. From a consideration of the relative effectiveness of the methyl groups on the pyrrole ring and of the benzene nucleus of indole, it was concluded that the indole-serine coupling enzyme combines with indole through the indole nitrogen. The utilization of tryptophane would seem to involve combination with the enzyme through the alanine side chain (18).

5-Methyl tryptophane exerts a strong inhibition on the growth of *E. coli* which is relieved by added tryptophane (156). It is interesting, however, that this antagonist acts like tryptophane in enhancing the adsorption of certain strains of bacteriophage on *E. coli* (156, 157). Indole inhibits the adsorption cofactor activity of tryptophane and phenylalanine in the above system in a manner suggesting competitive inhibition (157).

Evidence that anthranilic acid is an intermediate in the formation of indole and tryptophane is afforded by the fact that 4- and 5-methylanthranilic acids are potent inhibitors of the growth of *B. typhosum*, and that this inhibition is reversed by anthranilic acid, by indole, or by tryptophane (158).

The suggestion that conversion of tryptophane into nicotinic acid in the rat was inhibited by indole-3-acetic acid (159) has not been substantiated by further experiments (160, 161, 162).

Mutual antagonism between naturally-occurring metabolites.—It has long been evident that naturally-occurring metabolic intermediates or substrates may compete with other intermediates or substrates and thus become inhibitory. Some recent examples of this phenomenon are: the antimethionine action of norleucine in *E. coli* (16, 163) and *L. pentosus* (16); the antiglutamic acid effect of glutamine in *S. aureus* (164); the antiphenylalanine effect of tryptophane in *S. faecalis* R (12); the mutual and competitive antagonism of threonine and serine in a number of lactic acid bacteria (165); the antiserine effect of methionine in a methionine- and threonine-requiring mutant of *Neurospora* (166); the toxic effect on *L. arabinosus* and *Leuconostoc mesenterioides* of imbalances between leucine, isoleucine, valine, and methionine (167); the inhibition of the utilization of the pyrimidine ribonucleotides by a pyrimidine-deficient mutant of *Neurospora* exerted by adenosine or adenylic acid (168); and the antiphenylalanine effect of tyrosine

in *E. coli* (169). An interesting and somewhat similar situation is involved in a valine- and isoleucine-requiring *Neurospora* mutant in which a genetic block in isoleucine synthesis results in the accumulation of the corresponding keto acid which competitively inhibits the amination of the keto acid precursor of valine (170).

Miscellaneous inhibitors.—Granick & Gilder (171, 172) have studied the availability and antagonism of porphyrins to *Haemophilus influenzae* and have shown that porphyrins lacking vinyl groups were competitive antagonists of the growth-promoting prophyrins for this organism.

Trager (173) has observed that lysolecithin, which differs from lecithin in that it contains only saturated fatty acids, inhibits the growth of *L. casei*, this inhibition being reversed by increased amounts of biotin, of pantothenic acid, or by the addition of oleic acid or a neutral oil isolated from horse plasma. The results are consonant with the suggestion that lysolecithin may block lipid synthesis and that biotin and pantothenic acid are concerned in the synthesis of lipids.

The toxicity of fluoroacetate to animals and microorganisms appears to be due to a specific effect on the metabolism of acetate, probably by competitive antagonism (174, 175). The fluorine of fluoroacetate is, unlike that of its chloro-, bromo-, or iodo-analogues, very unreactive toward sulfhydryl groups, and was shown by Bartlett & Barron (174) to have no effect on a large number of enzyme systems. The immediate effect of fluoroacetate on the metabolism of acetate by yeast depends upon prior addition of the inhibitor (175), although with longer experimental periods the rate of acetate oxidation becomes constant and identical regardless of the order of addition (176). Studies by Kalnitsky on the effect of fluoroacetate on metabolism of kidney homogenates (177, 178) have shown that the metabolism of fatty acids, as well as of carbohydrates, may be inhibited. It is significant that the even-numbered omega substituted fluorocarboxylic acids were toxic to mice whereas the corresponding odd-numbered acids were not (179).

The insecticidal action of the hexachlorocyclohexanes has been shown to be due almost entirely to the gamma isomer (gam-mexane) (180). The growth of *S. cerevisiae* is inhibited by a hexachlorocyclohexane, the inhibition being competitively relieved by

meso-inositol (181). Schopfer *et al.* (182, 183) found that gammexane toxicity was reversed with some microorganisms and not with others. Further question as to the specificity of the gammexane-inositol antagonism is raised by the work of Chaix, Fromageot, and their associates, who find that the δ -isomer of hexachlorocyclohexane is more toxic than the γ -isomer to the ciliate *Glaucoma piriformis* (184), to the division of fertilized sea urchin eggs (185), and to a number of species of bacteria (186), *meso*-inositol being inactive in overcoming the effect of either isomer in any of these organisms. The toxic effect of gammexane on mosquito larvae was not influenced by *meso*-inositol (187). Although *meso*-inositol was able to reduce the inhibition of mitosis in onion roots produced by gammexane, it also reduced the inhibition brought about by the unrelated compound colchicine (188).

Woolley (189) has prepared several ethers of *N*-acetyldiiodotyrosine and found them to protect tadpoles against the lethal action of thyroxine. Frieden & Winzler (190, 191) have extended Woolley's observations and have shown that the *o*-benzyl ethers of diiodotyrosine and a number of analogues inhibit the effect of thyroxine on tadpole metamorphosis in a strictly competitive manner.

Trans-aconitic acid depressed the respiration of kidney slices, the inhibition being relieved by *cis*-aconitate, by citrate, or by succinate (192).

Stokstad and his collaborators (193) have reported that an enzyme from chicken pancreas which frees PGA from its conjugates is inhibited by *p*-aminobenzoyl- γ -glutamyl- γ -glutamyl-glutamic acid and its *p*-nitro analogue.

Wilson & du Vigneaud (194) have noted that penicillamine (β , β -dimethylcystine) given to rats on a low-choline diet, caused weight loss and convulsions, the effect being reversed by aminoethanol or its mono-, di-, or trimethyl derivatives, but not by methionine or cystine. Aminoethanol was most effective, suggesting that penicillamine exerts its toxic effect by blocking synthesis or utilization of aminoethanol.

No evidence supporting the suggestion that glucoascorbic acid is a metabolic antagonist of ascorbic acid was obtained by Gould (195), using the sensitive serum phosphatase test in guinea pigs as a measure of ascorbic acid activity.

The triethyl analogue of choline was shown by Keston & Wortis (196) to be acutely toxic to mice, the simultaneous administration of equal amounts of choline protecting. Working with isolated frog muscle, it was shown that triethylcholine blocked the contracting action of choline but not that of acetylcholine, suggesting that the choline analogue blocked the synthesis of acetylcholine.

Barron and his associates (197, 198) have indicated that the nitrogen mustards appear to inhibit reactions involving choline in a manner suggestive of a structural competition. Choline oxidase, acetylcholine esterase, and choline acetylase were much more sensitive to the nitrogen mustards than a large number of other enzymes that were tested, the inhibition appearing to depend in part upon the structural similarity of the ethylenimmonium derivatives to choline and acetylcholine. The effect of the nitrogen mustards, especially at higher concentrations, is also a function of their extreme reactivity with sulfhydryl groups and other groups essential for enzyme activity (198). β,β' -Dichloro-diethyl-N-methylamine has also been shown to inhibit the true choline esterase of tissues (199).

Concluding comments.—The usefulness of competitive antagonists of essential metabolites for studies of intermediary metabolism is becoming ever more apparent. The once puzzling fact that the effect of certain inhibitors can be overcome by structurally unrelated compounds has been developed, especially by Shive and his collaborators, into a useful method for elucidating metabolic pathways.

It is apparent from this survey of the literature that the advances which have been made have depended heavily upon the use of microorganisms. It is to be expected that as these and other metabolic antagonists are studied in animals and animal tissues, new observations of fundamental and practical significance are to be expected. Greenberg & Schulman (200), for example, have discussed the possible application of metabolic antagonists to the cancer problem. The problem of metabolic inhibitors, natural and synthetic, in nutrition is one which may also prove of considerable practical importance (201).

The question of what changes in structure are most likely to produce metabolic antagonists is one of considerable interest. The information that has accumulated since previous reviews does not

alter the general conclusions already reached (1, 4, 5, 6). It seems possible that many essential metabolites, especially those having a catalytic function, may have "combining groups" with which they associate with proteins and "functional structures" which are active in their catalytic roles. In such instances, it may be expected that alterations in the combining group may lead to inactive or less active compounds, while alterations in functional structure may yield antagonists (18, 191).

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CHEMISTRY OF ANTIBIOTICS

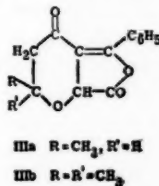
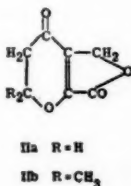
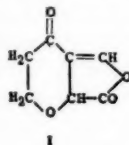
BY O. WINTERSTEINER AND J. D. DUTCHER

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Introductory remarks.—The vast volume of literature on antibiotics accruing since 1945, when the above subject was last reviewed by Oxford (1), has made it all the more necessary to adhere to the restrictive definition of the term antibiotic adopted by that author. Accordingly only factors derived from microorganisms and exhibiting reasonably high antimicrobial activity are considered, but the requirement that the antibiotic be known in the pure state was liberalized to accommodate certain polypeptide types. Even within this limited area complete coverage was not feasible, and discussion of individual topics had to be cursory. It is regretted that much meritorious work had to be omitted in order to keep within the space allowed, and that for the same reason, as well as by definition, the antimicrobial factors isolated from higher plants had to fall by the wayside. The classification by elementary composition in the order of increasing complexity as in Oxford's article has been retained, except that structurally closely related compounds have been grouped with the main representative regardless of composition; doubtful cases have been placed wherever they seemed to fit in best on the basis of general similarity of chemical properties or of microbial origin. A section dealing with chemical studies on the mode of action concludes the review.

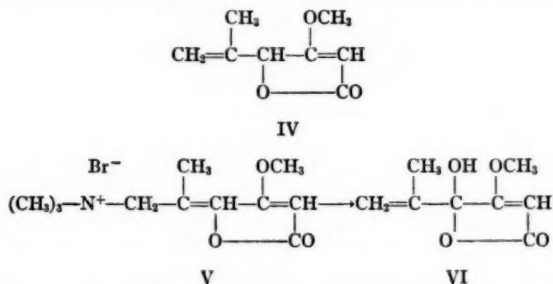
COMPOUNDS CONTAINING CARBON, HYDROGEN, AND OXYGEN

Clavacin [*Patulin*, *clavatin*, *claviformin*, *expansin* (2)], $C_7H_8O_4$.—Some new acyl derivatives of clavacin have been described (2). Existing uncertainties (1) regarding the correctness of the originally assigned structure I have not been resolved, but the synthesis



of a compound ascribed the alternative structure IIa has been reported (2). IIa is not identical with clavacin, is more stable than the latter, and in solution does not seem to exist in tautomeric equilibrium with I, as has been suggested (1). The close similarity of the absorption spectra of IIa, its homologue IIb, and of clavacin raises the question whether the latter is correctly represented by I with its lesser degree of conjugation. IIa and b showed only negligible bacteriostatic activity (3), as do the phenyl-substituted compounds IIIa and IIIb (4). This is explained (4) by the absence of hydrogen atoms at the β -position of the α, β -unsaturated keto grouping (cf. p. 585). However, it should be pointed out that the mode of preparation of IIIa and IIIb does not exclude the isomeric structure corresponding to type II. [For sources and biological properties of clavacin cf. (1)].

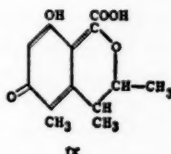
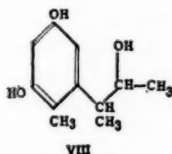
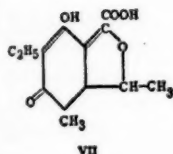
Penicillic acid, $C_8H_{10}O_4$ (1).—The total synthesis of this doubly unsaturated lactone (VI) has been achieved (5). The conversion of the key intermediate IV to VI was brought about by an interesting four-step sequence in which the quaternary base V is the immediate precursor of VI. It was furthermore demonstrated



by spectral measurements that penicillic acid even in alkaline solution exists in the lactol form VI, so that no tautomeric equilibrium with the open γ -keto acid form needs to be assumed.

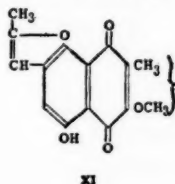
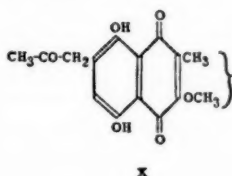
Gladiolic acid (*Penicillium gladioli*), $C_{11}H_{10}O_6$.—The chemical properties of this crystalline mold product suggest that it is methoxymethyl-2-carboxyphenylglyoxal, $\text{CH}_3\text{OCH}_2 \cdot \text{CO} \cdot \text{CO} \cdot \text{C}_6\text{H}_4 \cdot \text{COOH}$ (6). It is only weakly inhibitory for *S. aureus* (250 μg . per ml.) but has considerable fungistatic activity, 2 μg . per cc. preventing the germination of *Botrytis allii* conidia at pH 3.5.

Citrinin, $C_{13}H_{14}O_6$ (1).—Recent investigations have led to a



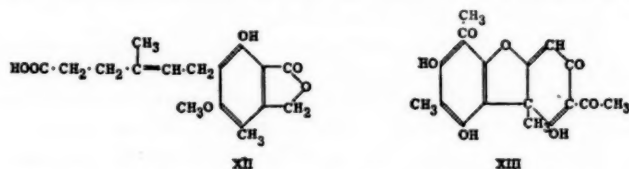
revision of the formula (VII) originally proposed (1). Doubts were first voiced by Gore *et al.* (7a) on the grounds that citrinin and the neutral, optically active degradation product $C_{11}H_{16}O_3$, which was assumed to be 2-ethyl-4-methyl-5-(1-hydroxyethyl)resorcinol (1), on coupling with diazonium salts yielded normal mono- and bis-azo dyes, respectively. A further degradation product, the dihydric phenol $C_9H_{12}O_2$ derived from the compound $C_{11}H_{16}O_3$ by alkali fusion and originally formulated as 2-ethyl-4-methylresorcinol (1), was shown by synthesis (8) and a study of the properties of its bis-benzeneazo derivative (7b) to be actually 4-methyl-5-ethylresorcinol. Its precursor $C_{11}H_{16}O_3$ can now with reasonable certainty be assigned structure VIII, accounting for its optical activity and other properties (8). Citrinin, on the basis of prior arguments as to the attachment of the remaining two carbon atoms, could then be written as IX (7b); but more recent evidence, including partial synthesis, appears to establish the structure which differs from IX in that the carboxyl group is allocated to the free position in the quinoid ring (9).

Javanicin (*Fusarium javanicum*), $C_{18}H_{14}O_8$.—This substance inhibits *S. aureus* at 1:400,000, and also shows considerable activity *in vitro* against *M. phlei* and *M. tuberculosis* (human strain), but affords little protection against the latter *in vivo* at the maximal tolerated dose (10). The proof for the proposed 1,4-naphthoquinone structure X (11) cannot be rendered in full here. The presence of this nucleus, and the substitution with hydroxyls in positions 5 and 8 appear well documented, salient points being the near coincidence of the absorption spectrum with that of hydroxy-



droserone (2-methyl-3,5,8-trihydroxy-2,4-naphthoquinone) and the behavior in certain color reactions indicating the absence of β -hydroxyl groups. The nature of the remaining substituents is deduced, *inter alia*, from analytical results (OCH_3 , two C-methyl groups), the positive iodoform reaction, and the formation of a dinitrophenylhydrazone and of an anhydro derivative, anhydro-javanicin (XI), which forms a monoacetate, whereas javanicin can accommodate two acyl groups. Anhydrojavanicin and its acetyl derivatives are antibiotically active, as is *oxyjavanicin*, $\text{C}_{18}\text{H}_{14}\text{O}_7$, a closely related metabolic product accompanying javanicin (10). Nevertheless, some specific dependence of the antibiotic activity on structural features present in the above type is evidenced by the relative ineffectiveness towards *M. phlei* of hydroxydroserone.

Mycophenolic acid (*Penicillium brevi-compactum* Dierckx), $\text{C}_{17}\text{H}_{20}\text{O}_7$.—This substance has only recently been recognized as an antibiotic agent (12), although its isolation from mold cultures dates back many years (cf. 12). The complete elucidation of its structure is likewise a late accomplishment (13). Structure XII is favored over the one in which the positions of the nuclear methyl group and of the seven-membered side chain are reversed. Mycophenolic acid is moderately active against gram-positive organism and displays some inhibiting power towards pathogenic fungi.



Usnic acid (*Cetaria islandica* Ach. and many other lichen species), $\text{C}_{18}\text{H}_{16}\text{O}_7$.—This compound has recently been recognized as a powerful inhibitor of mycobacteria, including the human strain of *M. tuberculosis*, as well as of some nonacid fast bacteria (14, 15). The D, L, and DL forms, all three of which occur in nature, are about equally effective. Several other lichen acids show similar antibacterial properties (14). The structure of usnic acid (XIII) may be considered as established (16, 17).

Viridin, $\text{C}_{20}\text{H}_{16}\text{O}_6$.—This substance is produced by certain pigment-forming strains of *Trichoderma viride*, while other strains

yield gliotoxin (18). It is characterized by the none too common property of being highly toxic to fungi without affecting the growth of bacteria (18, 19). Its fungistatic potency is 600 times that of gliotoxin with *Botrytis allii* conidia as the test organism, the minimum inhibiting concentration being 0.006 $\mu\text{g. per cc.}$ However, other fungi, including *T. viride*, do not show this high degree of susceptibility. Viridin is a colorless, crystalline, strongly dextrorotatory substance soluble in most organic solvents. In neutral aqueous solution it is rapidly inactivated, and even in acidic media its stability is very limited. Information on its structure is as yet fragmentary (19). Two biologically inactive hydrogenation products, $\text{C}_{20}\text{H}_{22}\text{O}_6$ and $\text{C}_{19}\text{H}_{24}\text{O}_4$ (the latter, a violet pigment, lacks a methoxyl group originally present), have been obtained. The facts so far ascertained can be expressed in the partial formula $\text{C}_{16}\text{H}_3(\text{COOCH}_3) \cdot (\text{OCOCH}_3) \cdot (\text{OH})_2$.

Pleurotin (Pleurotus griseus), $\text{C}_{20}\text{H}_{22}\text{O}_6$.—This yellow, crystalline compound, isolated by chloroform extraction from the culture filtrates of a basidiomycete, is inhibitory for gram positive bacteria and certain fungi (20). It reacts with potassium cyanide solution to give a blue color which serves as the basis of a chemical method of assay (21).

Fumigacin [Helvolic acid], $\text{C}_{32}\text{H}_{44}\text{O}_8$ (1).—Besides a paper (22) largely confirmatory of previous work there is to be mentioned a preliminary report (23) describing a hexahydro and an octahydro derivative of fumigacin, in which the original carbonyl function has been reduced to carbinol. It was also found that fumigacin as well as the hexahydro derivative reacts with three moles of alkali with the liberation of two moles of acetic acid.

Glutinosin (Metarrhizium glutinosum S. Pope) $\text{C}_{48}\text{H}_{60}\text{O}_{16}$ (24, 25).—This antibiotic resembles viridin in that its action is directed primarily against fungi and not bacteria, though quantitatively its fungistatic effectiveness does not approach that of viridin. As with the latter a high degree of specificity is apparent, which even extends to closely related species. Glutinosin is a dextrorotatory, high melting ($>300^\circ$) substance which in contradistinction to viridin is stable in acidic and slightly alkaline aqueous media. Further chemical data are lacking.

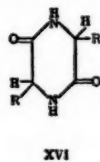
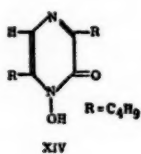
Antibacterial lipids.—The antibacterial activity of this group has been traced in all cases to a fatty acid component; it rests on the detergent properties of the latter and hence is not manifest

in vivo. *Pyolipic acid*, the amorphous but homogeneous material obtained by alcohol extraction of the bacterial cells of *Pseudomonas aeruginosa* (26), exhibits a high degree of *in vitro* activity against *M. tuberculosis*. Structure studies have shown it to be a conjugate of L- β -hydroxydecanoic acid, $C_7H_{15} \cdot CHOH \cdot CH_2 \cdot COOH$, and L-rhamnose (27). Two other instances of the isolation from microorganisms of lipids inhibiting acid-fast bacteria are on record. A mixture of saturated and unsaturated C_{22-24} fatty acids was obtained from the cell bodies of *Tetrahymena geleii* (28), and linoleic acid was found to be the chief constituent of an active fraction isolated from the mycelium of *Penicillium crustosum* (29).

COMPOUNDS CONTAINING CARBON, HYDROGEN, OXYGEN, AND NITROGEN

Aspergillic acid, $C_{12}H_{20}O_2N_2$ (1).—Since 1944 a number of publications dealing with the production and structure of this antibiotic have appeared (30 to 35). Woodward (30) has devised an improved medium suitable for large scale production of aspergillic acid which avoids the production of difficultly separable mixtures of aspergillic acid and hydroxyaspergillic acid such as were encountered by Bush *et al.* (36).

The structure of aspergillic acid, XIV, has been elucidated (31, 32, 33) by means of a series of degradations involving (a) reductive removal of the hydroxyl group on the nitrogen atom to form the neutral desoxyaspergillic acid, XV; (b) proof for the presence of a pyrazine nucleus by ultraviolet absorption studies and reduction with sodium and alcohol to a piperazine derivative; and (c) determination of the number and nature of the side chains by conversion to a 2,5-diketopiperazine, XVI, closely resembling isoleucine anhydride in its properties. Because of the complicated



stereochemistry involved, the exact identity of XVI was not established by synthesis, and recent work (35) has shown the need for revision of the assumption that both side chains are *sec*-butyl

groups. It was found that by hydrolytic cleavage of the diketopiperazine both iso- (or *alloiso*-) leucine and leucine are formed, and hence one of the R groups must be $-\text{CH}_2\cdot\text{CH}(\text{CH}_3)_2$ while the other is $-\text{CH}(\text{C}_2\text{H}_5)(\text{CH}_3)$. The respective position of these groups in aspergillic acid remains to be determined.

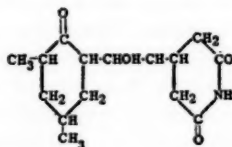
Hydroxyaspergillic acid, $\text{C}_{12}\text{H}_{20}\text{O}_3\text{N}_2$.—Hydroxyaspergillic acid, produced by the mold when growing on media rich in carbohydrate (37), differs from aspergillic acid by its considerably lower antibacterial activity. Contrary to earlier views the additional hydroxyl group is situated not on the pyrazine nucleus, but on one of the side chains, probably at the tertiary carbon atom of the *sec*-butyl group, since it could be readily eliminated by dehydrating agents. Since the compound $\text{C}_{12}\text{H}_{18}\text{O}_2\text{N}_2$ thus formed (dehydroaspergillic acid) could be reduced to products also obtained by reduction of aspergillic acid itself, there can be no doubt as to the presence of a common carbon and nitrogen skeleton (38).

Several synthetic compounds containing the cyclic hydroxamic acid grouping characteristic for aspergillic acid have been prepared and found to possess considerable *in vitro* antibacterial activity (39, 40).

The inappropriate designation of a number of different antibacterial agents produced by *Aspergilli* as "aspergillin" has inevitably resulted in confusion, the more so as the legitimate contender for this name by prior use is a pigment produced by *A. niger* (41).

Actidione (*Streptomyces griseus*), $\text{C}_{16}\text{H}_{18}\text{O}_4\text{N}$ (42).—The simultaneous production of streptomycin and actidione is but another instance of the now well-recognized ability of many microorganisms to elaborate two or more antibiotics entirely dissimilar in their chemical and antimicrobial properties. Actidione is much less hydrophilic than streptomycin and hence amenable to extraction by organic solvents. It inhibits the growth of many yeasts but has no effect on bacteria. Its toxicity to mammals varies widely for different species (LD_{50} 2.5 to 150 mg. per kg.). A ketone group is present (oxime, semicarbazone, the latter exhibiting one-twentieth of the activity of actidione). The biologically inactive monoacetate is an O-acetate. Structure XVII is proposed (43) on the basis of the degradation by alkali to ammonia, 2,4-dimethylcyclohexanone, and an aldehyde which on oxidation affords methanetriacetic acid. This acid is also formed directly on alkaline

cleavage of the diketonic oxidation product of actidione, dehydro-actidione.



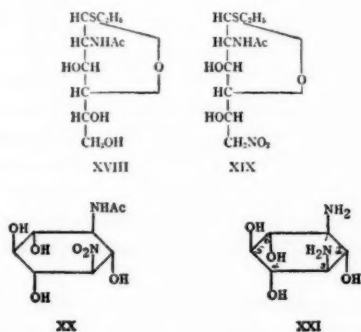
XVII

Streptomycin (*Streptomyces griseus*), $C_{21}H_{39}O_{12}N_7$.—The facts pertaining to the early history, and to the antibacterial, pharmacological, and therapeutic properties of this important antibiotic, discovered by Waksman and his colleagues in 1944, are now so well known as to require no elaboration here. The chemical work up to 1948 has been summarized in last year's volume of this *Review* (44) and in a detailed article by Lemieux & Wolfrom (45), which also treats other aspects such as isolation and chemical assay methods. Therefore, only additional material published during 1948 is considered here.

The trihydrochlorides of streptomycin and of mannosidostreptomycin (*vide infra*) have been obtained in crystalline form as the dihydrates (46). Counter-current distribution experiments have revealed that streptomycin exists in two or more tautomeric forms, which can be partially separated by chromatography (47). The free aldehydo group must be involved, since dihydrostreptomycin does not show this phenomenon.

O-tetramethylstreptamine has been degraded with permanganate to DL-dimethoxysuccinic acid, which proves that the 5-hydroxyl group in the parent diamine is oriented *trans* with respect to the 4- and 6-hydroxyl groups (48). Subsequently Wolfrom & Olin (49) announced the synthesis of streptamine from D-glucosamine by a route which at the same time completely elucidated the stereochemistry of this *meso*-compound. Ethyl 2-acetamido-2-desoxy- α -D-glucothiopyranoside (XVIII), accessible from pentaacetyl-D-glucosamine diethyl thioacetal by O-deacetylation and partial demercaptalization, was degraded with lead tetraacetate to the corresponding 5-aldehyde. The latter on condensation with nitromethane yielded two nitro derivatives, obviously ethyl 2-acetamido-6-nitro-2,6-didesoxy- α -L-idothiopyranoside (XIX) and

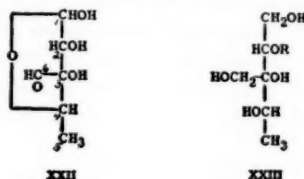
its 5-epimer possessing the D-glucose configuration. The N-acetyl-6-nitro-6-desoxy-L-idose (or D-glucose) resulting from the removal of the ethyl thio group from one of these compounds was cyclized to XX, which on reduction and acetylation yielded the known hexaacetylstreptamine. Streptamine is therefore represented as XXI, with all vicinal substituents *trans* to each other. The assumption that the two carbon atoms linked in the cyclization to XX (1 (3) and 2 in streptamine) have the configurations shown derives from evidence adduced by Grosheintz & Fischer (50) in the nitrodesoxyinositol series, indicating that in this type of condensation the nitro group is guided into *trans*-position with respect to the neighboring hydroxyl groups, and from the *meso*-character of streptamine. [The *trans*-arrangement would result regardless of whether the nitro sugar subjected to the cyclization was derived from XIX or from the corresponding D-glucose derivative, cf. (50)]. The same steric structure must be assigned to streptidine, the diguanido base corresponding to XXI. Its synthesis from streptamine by means of S-methylthiopseudourea (51) or cyanamide (52) has been accomplished.



The disclosure that heptabenzoylstreptidine, resulting from the hydrolysis of fully benzoylated streptomycin, is optically active (53) conclusively establishes position 4 (6) as the point of attachment of the streptobiosamine moiety and disposes of the objection (45) that benzoyl migration from C₄ to C₅ may have occurred during the hydrolysis, because in this case a racemic product should have been obtained (54). The same conclusion was reached by comparison of the periodic acid uptake of "decaacetyl-

dideguanidyldihydrostreptomycin," N,N'-diacetylstreptomine and methyl N-acetyl-dihydro- α -streptobiosaminide (55). Since the amorphous product containing the free "dideguanidyldihydrostreptomycin" (dihydrostreptobiosaminidostreptomine) was biologically inactive, it is evident that the guanido groups are essential for activity.

The steric positions of the 2- and 3-hydroxyl groups in streptose (XXII), the *cis*-relationship of which had been previously established (44), has been deduced from the application of Hudson's lactone and amide rules of rotation to derivatives of streptosonic acid and dihydrostreptosonic acid (56). More direct proof for the absolute configuration of C₂ was furnished by the degradation of N-acetyltetrahydrostreptobiosamine (XXIII) with periodic acid, followed by hydrolysis, to L-glyceric acid, formaldehyde, acetalde-



R = N-acetyl-N-methyl-L-glucosaminido

hyde, and N-methyl-L-glucosamine (57). Since the configuration of C₄ is known to be L (44), streptose is a 3-C-formyl-5-desoxy-L-lyxose (XXII). Thus the structure of streptomycin has been elucidated in all its stereochemical details.

Maltol, the product formed from the streptose moiety on exposure of streptomycin to alkali, has been synthesized from pyromeconic acid (58).

The fluorometric method previously described (44) has been adapted for the determination of streptomycin in tissues (59).

Mannosidostreptomycin (*Streptomyces griseus*), C₂₇H₄₉O₁₇N₇ (44, 45).—This term, now agreed upon to replace the original designation streptomycin B (60), takes into account the results of recent degradation work by Fried & Stavely (61) showing that the D-mannose is attached to carbon atom 4 of the N-methyl-L-glucosamine portion. First the sequence of the building stones in the molecule was ascertained by stepwise degradation of dihydromannosidostreptomycin to streptidine and a trisaccharide yielding

on methanolysis known methyl glycosidic derivatives of dihydrostreptobiosamine and D-mannose, and further to a disaccharide composed of N-methyl-L-glucosamine and D-mannose. The proof for the point of attachment of the latter to the former moiety was brought about by exhaustive methylation of N-pentaacetyldihydromannosidostreptomycin followed by hydrolysis. Identification of the definitive degradation products eventually obtained, namely 1,2,4-triacetyl-3,6-dimethyl-N-methyl-L-glucosamine and 3,6-dimethyl-L-glucosazone, was achieved through synthesis of the D-enantiomorphs by unambiguous methods. Evidence for the pyranose character of the N-methyl-L-glucosamine and D-mannose moieties in the intact antibiotic was adduced by periodic acid titration experiments. That the trisaccharide is attached to position 4 (6) of streptidine as in streptomycin was shown more recently by Peck *et al.* (62) by means of the benzylation method previously applied to the latter, and by Perlman & Langlykke (63), who were able to convert mannosidostreptomycin to streptomycin by enzymatic means.

The isolation of mannosidostreptomycin has been described in detail (64). Streptomycin and mannosidostreptomycin can be differentiated by paper strip chromatography (65). The existence in crude preparations of at least three additional active components is postulated.

Streptothricin (Streptomyces lavendulae).—This antibiotic resembles streptomycin in that its action is directed primarily against gram negative and acid-fast organisms (66), but differs from it by its higher toxicity. For the latter reason little attention has been paid to this interesting substance since its early isolation in form of the crystalline reineckate (67, 68) and helianthate (68). The few chemical data reported, including the elementary composition $C_{13}H_{25}O_7N_6$, are to be regarded as tentative (67). The negative Sakaguchi reaction for guanido groups, and the positive biuret and ninhydrin tests make close relationship to streptomycin doubtful. Very similar to streptothricin in regard to source and antibacterial properties are lavendulin and actinorubin both of which have been isolated as the crystalline helianthates (69). In the absence of further chemical characterization, the contention that these antibiotics are distinct entities, both closely related to, but not identical with, streptothricin, rests on certain discrepancies in the analytical composition of the helianthates,

and differences in the quantitative response of a few test organisms (70). In the case of streptolin (71), which was likewise isolated via the helianthate, identity with streptothricin, in spite of general similarity of chemical and antibacterial properties, is definitely excluded by marked differences in the rotation values and in the minimal concentrations inhibiting various susceptible bacteria. The source organism, an unidentified *Streptomyces* species, also produces streptothricin, which, however, was separable from streptolin by adsorption procedures (72). Finally, the recently described xanthomycins A and B (73), also from an unidentified *Streptomyces* species, may be counted to this group insofar as they are basic substances amenable to purification via the picrates or reineckates. In most other respects, however, there is but little similarity: they are yellow pigments and markedly organophilic in the form of the free bases; their high inhibitory potency towards both gram negative and gram positive bacteria, but above all their extreme toxicity, puts them into proximity to actinomycin rather than to streptothricin. The A and B factors are closely related in their properties, but separable by Craig distribution. Only xanthomycin A could be obtained in crystalline form as the reineckate.

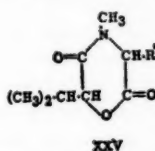
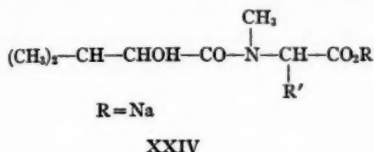
Enniatin [Lateritiin] and related products from Fusaria, $C_{22}H_{38}O_6N_2$ and $C_{24}H_{42}O_6N_2$.—This group of neutral, crystalline compounds isolated from the mycelium of various species of *Fusaria* is characterized by high *in vitro* activity against *M. tuberculosis* and low toxicity, but possesses negligible therapeutic value (74).

Recent studies by Plattner & Nager (75 to 78) have made it clear that what were originally thought to be well-defined members of this group are in reality difficultly separable mixtures. At the present time three distinct compounds, enniatin A, B, and C, would appear to account for all the recorded members of this group of antibiotics, although additional compounds are not excluded.

Since the lateritiin I of Cook *et al.* (74) also proved to be a mixture of enniatins A, B, and C (78), it appears probable that the other members of that series, lateritiin II, avenacein, fructigenin, and sambucinin, may be accounted for by the same products.

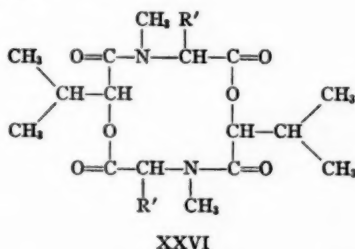
Both groups of workers have obtained and identified by synthesis the products of acid and alkaline hydrolysis. In all cases acid

hydrolysis yields two moles of D(-)- α -hydroxyisovaleric acid (75, 79) and in addition two moles of an N-methylated α -amino acid, the nature of which characterizes the different entities; enniatin A yields N-methyl-L-isoleucine; enniatin B, N-methyl-L-valine; and enniatin C, N-methyl-L-leucine. Hydrolysis with two equivalents of alkali affords a product of structure XXIV ($R = Na$) which can be isolated in the form of either the ester or the lactone, XXV.



Enniatin A: $R' = \text{CH}(\text{CH}_3)(\text{C}_2\text{H}_5)$
 Enniatin B: $R' = \text{CH}(\text{CH}_3)_2$
 Enniatin C: $R' = \text{CH}_2\text{CH}(\text{CH}_3)_2$

On the basis of these degradations the Swiss workers advance structure XXVI as accounting best for the known properties of these products (76).



Pyo compounds (Pseudomonas aeruginosa).—This bacillus has been known to produce at least two well-defined antibiotically active substances, the phenazine derivatives pyocyanine and α -hydroxyphenazine (1, 80). To these has now been added a series of crystalline antibiotics of unknown, but apparently more complex structure—the pyo compounds (81). They were obtained from the cells of a selected strain by alcohol extraction and separated from each other by a primary separation into a carbonate-soluble fraction containing Pyo II, $\text{C}_{34}\text{H}_{46}\text{O}_4\text{N}_2$, and a neutral fraction yielding Pyo I, Pyo III, $\text{C}_{34}\text{H}_{44}\text{O}_2\text{N}_2$, and Pyo IV, $\text{C}_{16}\text{H}_{23}\text{O}_3\text{N}$, which in turn are separated by chromatography on permutit. Pyo

I was found to be a mixture consisting of Pyo Ib, $C_{31}H_{40}O_2N_2$, and Pyo Ic, $C_{34}H_{48}O_2N_2$. Except for Pyo II, which is light yellow, all the compounds are colorless, but show intense and characteristic absorption in the ultraviolet region. Attempts at characterization by derivatives were generally unsuccessful, except with Pyo IV, which forms a dibenzoate and a 2,4-dinitrophenylhydrazone. However, characteristic hydrogenation products could be obtained from all but Pyo IV, and structural relationships are apparent from the identity of tetrahydro Pyo III with Pyo Ic, and of octahydro Pyo Ic with dodecahydro Pyo III. Ozonolysis (82) of Pyo III yielded *n*-octanal, while Pyo Ic was thus degraded to *N*-capryl-anthranilic acid, capric acid, and capramide. In general, the Pyo compounds are markedly inhibitory only for gram positive bacteria (81). Preliminary data indicate that Pyo II, which shows the highest *in vitro* potency, is fairly toxic and incapable in sub-toxic doses of protecting mice infected with *Diplococcus pneumoniae* or *M. tuberculosis* (83).

Actinomycin (Actinomyces antibioticus), $C_{41}H_{66}O_{11}N_8$ (1).—The name actinomycin A should be dropped, since the existence of actinomycin B as a separate entity now appears highly doubtful (84). Actinomycin has been isolated from cultures of *Actinomyces parvus* Kraisky (85). No new facts pertaining to structure have been forthcoming.

Aureomycin (Streptomyces aureofaciens sp. n.).—No chemical information is available on this important new antibiotic other than that it is a yellow crystalline, amphoteric substance stable in solution at pH 2.5 but not at pH 8.5.¹ However, its antibacterial, pharmacological and therapeutic properties have been described in detail (86); and there is ample clinical evidence on record attesting its effectiveness particularly in human rickettsial diseases.

COMPOUNDS CONTAINING CARBON, HYDROGEN, OXYGEN, NITROGEN, AND SULFUR

Gliotoxin, $C_{13}H_{14}O_4N_2S_2$ (1).—This substance has proved to be one of the most ubiquitous among the known antibiotics. Since its discovery by Weindling in 1936 (87) in the culture filtrate of an organism then classified as *Trichoderma lignorum*, its production by no less than 10 different organisms has been reported. Brian (88) has questioned the original and modified classification of

¹ [See 86, p. 183]

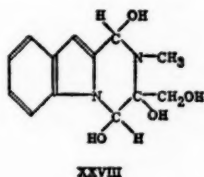
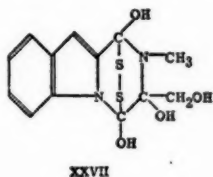
Weindling's organism and presents evidence in favor of reclassifying it as a strain of *Trichoderma viride*.

The following other organisms have been shown to yield this compound: *Aspergillus fumigatus* (1, 89); *Penicillium terlikowskii* (90); *Penicillium jensenii* (91); *Penicillium obscurum* Biourge, which also produces a compound, probably $C_{14}H_{16}O_4N_2S_2$, related to gliotoxin but only one-tenth as active (92); *Penicillium cinerascens* Biourge (93); *Penicillium terrestre* Jensen (?) and five other Australian species of *Penicillia* (94). These latter species produced a crystalline product which the Australian workers called "penicidin" but from their description of its properties there seems little doubt but that the material in hand was gliotoxin.

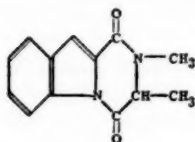
Gliotoxin has a broad range of antibiotic activity; the list of organisms and tissues affected by it ranges from pathogenic fungi (95) and bacteria (96) to *M. tuberculosis* (97) and even includes the malarial organisms (98, p. 910) and cancer tissue (99). In spite of high *in vitro* activity, it has not proved of therapeutic value because of its high toxicity.

A series of papers dealing with the structure of gliotoxin has appeared (100 to 104), but the complexity of the molecule has so far prevented complete elucidation of its constitution.

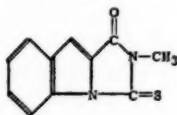
The main structure features are apparent in formula XXVII, one of the alternative structures proposed. The existence of the disulfide linkage follows from the formation, on treatment with various reductants, of a dimercapto form (104), a reaction which appears to be the basis for the reversible inactivation by cysteine (105). Aluminum amalgam effects the complete removal of the sulfur atoms and leads to the formation of desthiogliotoxin,



$C_{13}H_{16}O_4N_2$, presumably of structure XXVIII (104). Proof of the nature of the carbon and nitrogen skeleton rests on the reduction of gliotoxin with hydriodic acid to the product $C_{13}H_{12}O_2N_2$, XXIX, the structure of which has been established by total synthesis



XXIX



XXX

(101). One of the products formed by mild alkaline hydrolysis of gliotoxin has been shown to be the thiohydantoin XXX by conversion via the corresponding hydantoin to indole-2-carboxylic acid (106), and by total synthesis (227).

Penicillin.—Only recent contributions not covered by the excellent review of Chain (107) and some previous work on aspects not treated there are considered in this section.

Isolation.—The penicillin-like factor produced by *Aspergillus parasiticus* (parasitacin) has been identified as benzylpenicillin (108). A reinvestigation of "flavacidin" from *Aspergillus flavus*, formerly believed to be 3-pentenylpenicillin (109), has shown that this factor is actually *n*-amylpenicillin, formed by the mold along with benzylpenicillin and 2-pentenylpenicillin (110). It is interesting to note that *n*-amylpenicillin was first encountered in nature as a metabolic product ("gigantic acid") of *Aspergillus giganteus* (111), and only recently has been isolated from culture filtrates of a *Penicillium notatum* strain (112). Innate capacity to produce penicillins of various kinds therefore seems to be more widely distributed among *Aspergilli* than among *Penicillia*.

Biosynthesis.—Extension of the work with precursors by Behrens and his colleagues (113) has led to 19 new biosynthetic penicillins in addition to the 11 previously reported (107), attesting anew the remarkable ability of the mold to utilize a great variety of acyl groups offered in form of the acids or amides as building stones for the side chain. In most of these new penicillins the specific R-group has the structure $R'SCH_2-$, in which R' may be alkyl, aryl (for instance 5-thienyl), or aralkyl, in some cases carrying halide substituents [for $R' = CH_2CCl = CHCH_2-$, cf. (114)]. A selenium-containing penicillin, with $R = C_6H_5SeCH_2-$, was also prepared. Another interesting member of this group is cyclopentylmethylpenicillin, which represents the closest approximation to the "natural" aliphatic types so far realized by the precursor method.

Other papers by Behrens *et al.* deal with methods for the evaluation of precursor materials for benzylpenicillin (115) and for new penicillins (116). It was also shown by the use of deuterophenylacetyl-N¹⁵-DL-valine that while the mold readily utilized the acyl group in the biosynthesis of benzylpenicillin, the valine nitrogen was not incorporated (117).

Chemistry.²—The alcoholysis of penicillin is greatly accelerated by, and in practice dependent on, catalysis by metal ions (118). Benzylpenicillinic acid (free benzylpenicillin) has been obtained in form of stable, nonhygroscopic crystals containing one molecule of isopropylether, while other "natural" penicillins failed to yield crystalline addition products with this solvent (119). The (amorphous) anhydride of benzylpenicillinic acid (120), formed from the latter by the action of thionyl chloride, promises to be of value for arriving at hitherto inaccessible benzylpenicillin esters and amides. Thus the anhydride was used successfully for the preparation of the readily hydrolysable β -dimethylaminoethyl ester. Similarly, the mixed anhydride of benzylpenicillin and acetic acid has been prepared and converted into the crystalline, biologically active benzylpenicillin amide (121). While not differing from penicillin in regard to chemical stability, the amide is resistant to penicillinase.

Following the demonstration that the crude condensation product of D-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone on standing in ethanol solution gives a high yield of D-benzylpenillic acid (122), it has been shown by similar studies in the DL-series that this synthesis of the penillic acid involves D-benzylpenicillenic acid as an intermediate. The condensation reaction, when carried out with DL-penicillamine, yielded crystalline DL-benzylpenicillenic acid, which on treatment with ethanol partly rearranged into DL-benzylpenillic acid (123). Furthermore, the availability of the former acid in pure state, in conjunction with an ingeniously contrived proof for the identity of "natural" (i.e., formed by rearrangement of benzylpenicillin) and synthetic D-benzylpenicillenic acid (an amorphous product) (124), made it possible to demonstrate conclusively that the formation of the benzylpenicillin produced in small amounts in the condensation of D-penicillamine with the oxazolone occurs via its isomeride, the

² For the structure of the isomerides and degradation products of penicillin mentioned in this and the following section, cf. Chain (107).

D-penicillenic acid (123, 124). Several analogues of benzylpenicillin, as well as phenyl- and styrylpenicillin, have been synthesized in minute yields via the corresponding penicillenic acids (125).

The Raney nickel desulfurization of sodium benzylpenicillinate to desthiobenzylpenicillin, a key product in the proof for the β -lactam structure of penicillin (107), has been carried out under conditions sufficiently mild to render a rearrangement in this reaction quite improbable (126). On the other hand, the formation of α -phenyllevulinic acid in the degradation of benzylpenicillin by alkali is at variance with all previous evidence regarding the nature of the essential building stones, and must be explained on the assumption that some of the conventional degradation products enter secondary condensation reactions coupled with reductive processes (127).

Analytical.—The countercurrent distribution method of Craig as applied by that author to penicillin (128) has proved to be of great value for the purity control of crystalline preparations as well as for the analysis of penicillin mixtures. The effect on the experimental distribution curves of disequilibrium (129) and of time as related to the stability of the penicillins in various solvent-buffer systems (130) has been studied. The procedure can also be used for the isolation and identification of penicillins occurring in crude mixtures, as in the case of the S^{35} -containing benzylpenicillin (131) produced by biosynthesis in the presence of S^{35} labeled sodium sulfate (132). Partition chromatography, one of the earliest methods used for separating the various penicillins (133), has been adapted for the quantitative analysis of mixed penicillins (134). Paper strip microchromatography, in conjunction with biological assay, can also serve as a rapid, if quantitatively less precise tool for this purpose (135, 136). Furthermore, techniques employing infrared spectrophotometry for differentiating quantitatively benzylpenicillin in crystalline mixtures have been developed (137, 138). Among methods for the determination of benzylpenicillin in purified material, a chemical procedure based on the precipitation of the N-ethylpiperidine salt (139), and a colorimetric one employing a modification of the Kapeller-Adler reaction for phenylalanine (140) appear to be the most specific, while the ultraviolet assay (141, 142), since it is based on evaluation of the bands in the region 250 to 270 $m\mu$ originating in the phenyl group, presupposes the absence of contaminants containing aromatic

groups, for instance *p*-hydroxybenzylpenicillin. In culture broth benzylpenicillin can be determined by a method combining chromatography on Super-Filtrol with biological assay (143).

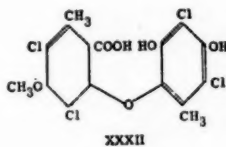
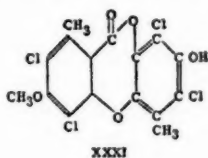
The chemical methods for the estimation of total penicillin depend either on alkaline or enzymatic hydrolysis to penicilloic acid, or the formation of penicilloic α -amides by reaction with suitably substituted amines. To the latter group belong the colorimetric (144, 145) and fluorimetric (146) procedures in which penicillin is transformed to chromophoric or potentially chromophoric amides. In the former group, penicilloic acid is determined either acidimetrically (147, 148), manometrically (149) or iodometrically (150, 151, 152). Of these the iodometric procedure recommends itself because of greater sensitivity and specificity, an advantage also claimed for the titration with alkali of an acidic entity (presumably the sulfonic acid corresponding to penicillamine, penicillaminic acid), liberated by treatment of penicillin with hydrogen peroxide (153).

A critical discussion of the principal chemical and spectrographic methods and of their limitations is available (154).

Sulfactin (*Actinomyces* sp., possibly *A. roseus*), $C_{38}H_{56}O_7N_{11}S_4$ (155).—This compound is produced by the mold on a starch-tryptone medium in shake culture, from the filtrate of which it is isolated by butanol extraction. Purification is effected by chromatographing on Florisil in chloroform solution. Sulfactin is described as a stable, colorless, crystalline substance soluble in most organic solvents except hydrocarbons and ether, and insoluble in water. The above formula is provisional. The published biological data are scant and reveal only that it is highly active against a few gram positive bacteria.

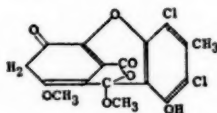
COMPOUNDS CONTAINING ORGANICALLY-BOUND CHLORINE

Diploicin, $C_{16}H_{10}O_5Cl_4$.—The isolation of this compound from the lichen *Buellia canescens* was reported by Nolan (156) in 1935. Subsequent work by this investigator and his colleagues (157) led

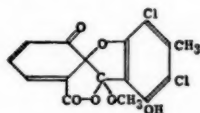


to its formulation as XXXI [but cf. also (158), p. 151]. The similarity of XXXI to certain chlorinated diphenyl ethers known to possess high tuberculostatic activity *in vitro* recently prompted Barry (159) to examine the derived acid XXXII for this property (diploicin itself could not be tested on account of its insolubility in water). XXXII was indeed found to inhibit the test organisms (*M. tuberculosis* and *M. smegmatis*) in moderately high dilutions.

Geodin (*Aspergillus terreus* Thom), $C_{17}H_{12}O_7Cl_2$.—This interesting substance, which had been isolated in 1936 by Raistrick & Smith (160), together with the closely related erdin, has only recently been shown to possess antibiotic properties (4, 161). It is moderately bacteriostatic for gram positive organisms, but was ineffective against most of the gram negative bacteria examined. Its inhibitory power towards *M. smegmatis* is apparently much weaker than that of diploicin. Resumption of earlier structure studies has now led Calam *et al.* (162) to the tentative formulation of geodin as XXXIII, a or b, and of erdin as the free carboxylic acid corresponding to these alternative *pseudo* ester structures. The distinguishing *pseudo* ester feature is postulated to account for the fact that geodin is optically active, whereas erdin is not, and furthermore that the two compounds on methylation with diazomethane yield isomeric but not identical products. It is also suggested that since erdin lacks antibiotic properties, the biological activity might be associated with this grouping.



XXXIIIa



XXXIIIb

Ustin, $C_{19}H_{16}O_6Cl_3$ (?) and other products from *Aspergillus ustus*.

—Following the demonstration that ether extracts of the filtrates of this mold are highly inhibiting to mycobacteria (*tuberculosis* and *rauae*) (163) Hogeboom & Craig (164) and Doering *et al.* (165) isolated several crystalline, apparently closely related, products which all exhibit this property. Identity of at least one of these, an acid designated Compound I by the former, and ustin by the latter authors, seems probable. Monomethyl and dimethyl derivatives, and an acetate have been prepared (165). Compound II of Hoge-

boom & Craig is likewise acidic, but somewhat less active than I, while the three other compounds described by Doering *et al.* are neutral, and in two instances indistinguishable from ustin in regard to potency. There is agreement that gram negative organisms are not affected by ustin, but statements regarding the susceptibility of gram positive cocci are seemingly at variance (163, 165). The fact that the addition of serum albumin all but abolishes the antibacterial effect (165) does not augur well for therapeutic usefulness.

Chloromycetin (Streptomyces venezuelae sp. n.) (166).—This chlorine-containing antibiotic has recently attracted considerable attention as it is one of the few chemotherapeutic agents found to be of value in the treatment of rickettsial diseases such as scrub typhus and typhus fever. Its isolation in crystalline form from cultures of a new species recently named *Streptomyces venezuelae* (166) has been reported by two laboratories (167, 168). Details of the simple solvent extraction procedures used and preliminary chemical data are given in a paper by Bartz (169). Chloromycetin is a colorless, neutral, levorotatory substance soluble in organic solvents except hydrocarbons, but also slightly soluble in water, and quite stable in acid and neutral, but not in alkaline, aqueous media. Though no empirical formula is assigned, the analytical data would seem to indicate $(C_{11}H_{12}O_5N_2Cl_2)$. Outstanding among its biological properties is its effectiveness in experimental rickettsial (170, 171) and viral (170) infections, but it also displays considerable *in vitro* activity against gram negative organisms, and to a lesser degree against gram positive and acid-fast bacteria (167, 168, 169). Other features favorable to therapeutic applicability are low toxicity and the fact that the compound appears to be well absorbed on peroral administration (171). It remains to be seen to what extent these properties can be correlated with chemical structure.

POLYPEPTIDES

Mainly in consequence of the renewed attention given to bacteria as sources of antibiotic agents this group now counts many more members than at the time of the last review, when only four antibiotics of this type had been recorded (gramicidin, tyrocidine, diplococcin, and notatin). While rigid criteria for chemical purity are as a rule lacking with this class, the characterization by chemical and biological properties is usually sufficiently precise to serve

as a basis for differentiation. Nevertheless the doubtful homogeneity of some of these substances must be kept in mind in evaluating the significance of the available chemical, potency and toxicity data.

Bacillin.—This name is given to an antibacterial factor isolated in crude form from culture filtrates of several strains of *Bacillus subtilis* (172). Its characterization rests entirely on biological data, particularly such serving to differentiate it from other factors of similar origin. Bacillin is unique in that its bacteriostatic action is completely neutralized by an antagonistic factor widely distributed in biological materials, including blood serum (173).

Bacitracin.—The source organism of this antibiotic is an as yet unidentified bacillus originally isolated from the infected tissue of a bone fracture case (174). Bacitracin is found in the filtrate of static cultures of this organism and can be extracted with *n*-butanol. Detailed information concerning the methods used for further purification and the degree of purity thus attained is not yet available. The antibacterial range of bacitracin is similar to that of penicillin, but its nephrotoxicity, albeit of a moderate degree, would seem to limit its use in systemic infections. However, with the commercially produced preparations this property seems to vary independently of antibacterial potency (175). That bacitracin has polypeptide character is evident from recently reported countercurrent distribution and starch column chromatographic studies (176). The major component demonstrable in the material examined by the former procedure contained 83 per cent of the solids and all of the biological activity; chromatography of the hydrolysate of this portion revealed the following amino acid composition (grams per 100 gm. substrate): phenylalanine, 11; leucine, 9; isoleucine, 22; glutamic acid, 10; aspartic acid, 17; lysine, 9; histidine, 10; cystine, 14. Definitely absent were methionine, valine, threonine, serine, proline, and arginine.

Diplococcin (1).—A new finding deserving mention is the demonstration that diplococcin exhibits inhibitory power *in vitro* for *M. tuberculosis* (177).

Eumycin (178).—The scant chemical and biological data so far recorded leave open the question whether this factor, which occurs in the broth of static cultures of a *B. subtilis* strain, should be regarded as an entity distinct from the other antibiotics elaborated by this species. The active agent is precipitated from the broth by

acidification and can then be extracted from the precipitate by ethanol, butanol or acetone. It is stable to acid but rapidly destroyed above pH 8. Eumycin is reported to be highly active *in vitro* against *M. tuberculosis* and to offer some protection to mice infected with a virulent strain of this microorganism.

Gramicidin S (gramicidin C).—A strain of *Bacillus brevis* isolated by Russian workers (179, 180) is the source of a tyrocidine-like substance which has been called gramicidin S (Soviet) (in initial reports, gramicidin C). The crystalline, levorotatory hydrochloride was obtained chiefly from the cells by a process entailing precipitation with acid and extraction with ethanol. The free peptide has likewise been secured in crystalline form. Gramicidin S is highly stable to heat; the crystals in the dry state withstand heating at 160°C., and the aqueous solution may be autoclaved at 120°C. for 30 min. Study of the hydrolytic products (181) has shown the stoichiometric minimum unit to embody one residue each of L-ornithine, L-proline, L-valine, L-leucine, and D-phenylalanine. Since the peptide contains no free carboxyl groups (181), and the only free amino group present is that in the δ -position of ornithine (182), it follows that gramicidin S is a cyclopeptide and hence very closely related indeed to tyrocidine. Examination, mainly by paper chromatographic methods, of the products of partial hydrolysis has shown that the sequence of amino acids in the closed chain is: $-\alpha$ -L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl- (183). Crystallographic studies favor a simple cyclopentapeptide, but a cyclic decapeptide structure, doubling the above sequence, is not excluded.

Gramicidin D (Dubos) (1, 184).—The hydroxyl-amino compound observed in hydrolysates of gramicidin D (184) has been identified as ethanolamine (185). This residue accounts for 3.2 per cent of the total nitrogen and completes the list of constituents of this antibacterial compound. Recent data by Synge [see (186), footnote p. 63] suggest a stoichiometric minimum unit, with a molecular weight of approximately 2,000, made up from four residues each of D-leucine, L-tryptophane, and valine (2D- and 2L-), two of L-alanine, and one each of glycine and ethanolamine. Since no free carboxyl or amino groups are demonstrable (184), gramicidin D would also appear to be a cyclopeptide.

Much attention has been paid to the problem of improving through chemical manipulation those properties of gramicidin D

which have so far militated against its therapeutic use in systemic infections, namely, its hemolytic activity, high toxicity, ineffectiveness in the presence of serum, and insolubility in aqueous media. Treatment with formaldehyde (187 to 190) diminished the hemolytic titer and toxicity to white cells without impairing the antibiotic activity. Other reagents which have been explored for this purpose are nitrous acid, chromic acid, bromine, iodine, and hydroxylamine (191); and also glyoxal, pyridine-chlorosulfonic acid, acetic anhydride, and succinic anhydride (188).

Licheniformin.—This antibiotic, produced by *Bacillus licheniformis* in static culture, resides for the most part in the cell bodies from which it has to be liberated by autoclaving at pH 2.5. After purification by adsorption on charcoal and precipitation with picric acid it is obtained in the form of an amorphous hydrochloride which is soluble in methanol, but not in ethanol or acetone, dialyses through cellophane, and gives positive biuret and Sakaguchi reactions (192). That preparations thus purified are not homogeneous is apparent from the inconstancy of the potency: toxicity ratio (193). Nevertheless, the available chemical and biological data suffice to differentiate licheniformin from the *B. subtilis* factors. The effectiveness of licheniformin in experimental *M. tuberculosis* infections is of interest, but its usefulness in this respect will largely depend on whether or not the rather marked nephrotoxicity exhibited by the preparations so far used can be eliminated by further purification.

Polymyxin (Aerosporin).—In 1947, Benedict & Langlykke (194) and Stansly, Shepherd & White (195) almost simultaneously put on record their discovery of an antibacterial factor produced by *Bacillus polymyxa* and named polymyxin by the latter authors. Meanwhile Ainsworth, Brown & Brownlee (196) had been studying "aerosporin," a substance isolated from the culture filtrates of an organism then classified as *Bacillus aerosporus*. It has now been shown (197) that these two factors are members of a group of closely related compounds, of which five, each produced by a different strain, have so far been characterized. In view of the fact that the organism previously called *B. aerosporus* has recently been identified as *B. polymyxa*, the generic name polymyxin has been adopted for this group of substances, the individual members being differentiated by the use of capital letters (198). Details of their isolation as amorphous hydrochlorides from the cell-free

filtrate of either static or submerged fermentation cultures have been recently communicated (199). The polymyxins have a wide range of antibacterial activity but are particularly effective against gram negative bacteria, including such pathogens as *Brucella abortus*, *E. coli*, *Hemophilus influenzae*, *Proteus vulgaris*, and *Salmonella enteritidis*. Very few, if any, resistant strains developed under the conditions of study, and the antibacterial effect persisted in the presence of blood serum (195).

The polymyxins are basic peptide-like compounds which on acid hydrolysis yield besides a mixture of amino acids an optically active fatty acid, $C_9H_{18}O_2$ (200). The individual members of the group can be readily differentiated by paper chromatography, which also served as the tool for determining their amino acid composition (197). They all contain L-threonine, L- α , γ -diaminobutyric acid and the saturated fatty acid mentioned above, and in addition one or two amino acids characteristic for the individual species. These differentiating entities are: in polymyxin A (aerosporin), D-leucine; in polymyxin C, phenylalanine; in polymyxin D (Stansly *et al.*), D-leucine and serine; and in polymyxin B, D-leucine and phenylalanine. In polymyxin E, as in A, the additional component is D-leucine, so that the distinction between these two factors rests solely on differences in their migration rates on the paper chromatogram. Polymyxin A and D are both nephrotoxic (201) while polymyxins B and E are reported to be free from this property (197, 200).

Subtilin.—This antibiotic is produced by a *Bacillus subtilis* strain in either static or submerged culture (202, 203). It is extractable by 65 per cent ethanol or aqueous butanol (204) but is inactivated by methanol. General instability, and the fact that the active material is appreciably soluble only at low pH and low salt concentration, rendered purification difficult. Recent preparations appear to be homogeneous as shown by electrophoretic analysis and their behavior in diffusion and precipitation procedures (205). Differentiation from the other peptide antibiotics has been achieved by comparison of the antibacterial spectrum and physicochemical properties (206). A distinguishing characteristic of the purified material is the high sulfur content (4.8 per cent). The molecular weight is given as about 6,500. The amino acid composition, on the basis of this figure, is as follows: 4 glycine, 9 alanine, 2L-valine, 2L-isoleucine, 8 L-leucine, 2 L-phenylalanine, 2 L-proline,

1 tryptophane, 5 L-lysine, 2 aspartic acid, 5 L-gultamic acid; the sulfur containing entities are lanthionine and another as yet unidentified compound, $C_7H_{14}O_4N_2S$. Phosphorus, sulfhydryl, disulfide, methio, methoxy and ethoxy groups are absent (207).

Subtilin inhibits, *in vitro*, a number of pathogenic organisms including *Bacillus anthracis*, *Diplococcus pneumoniae*, *Neisseria gonorrhoeae*, and *M. tuberculosis* (208). It affords protection to mice infected with *D. pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*; a suppressive effect on *M. tuberculosis* in guinea pigs (209) was likewise demonstrable. It has low toxicity (209).

Tyrocidine.—The free groups of this peptide [cf. (1, 184)] have been shown to be the δ -amino group of ornithine and the phenolic group of tyrosine (210). Hydrolysis, after treatment with S-methylisothiurea, yielded 1 arginine per 13 original nitrogen atoms. After reaction with *p*-toluenesulfonyl chloride only O-*p*-tosyl-tyrosine and δ -*p*-tosyl-ornithine could be detected in the hydrolysis products. No terminal α -amino or carboxyl groups could be demonstrated (210). These findings bring the structure of tyrocidine into close relationship to that of gramicidin S; both are cyclopeptides containing ornithine (found here for the first time as such in a protein or polypeptide) in which the δ -amino group is free; both contain D-phenylalanine while the other α -amino acid residues have the L-configuration; tyrocidine, however, has a greater and more varied complement of amino acids (3 D-phenylalanine residues and 2 residues each of L-ornithine, L-proline, L-valine, L-leucine, L-tryptophane, L-tyrosine, L-aspartic acid, and L-glutamic acid (184).

MODE OF ACTION

Most of the studies so designated deal with the influence of antibiotics on bacterial metabolism as a whole or some of its phases, and on specific enzymes known or presumed to be involved in these metabolic processes. For lack of space, as well as of competence in these matters, the reviewers have confined their attention to those few investigations in which the problem is approached primarily from the chemical side, i.e., where the properties and structure of the antibiotics themselves importantly enter the argument about the mode of action.

In 1944 Cavallito & Bailey (211), intrigued by the finding that

such chemically diverse types as penicillin, citrinin, gliotoxin, clavacin, pyocyanine, and the active agents from the green plants *Allium sativum* (later shown to be $\text{CH}_2=\text{CH}\cdot\text{CH}_2\cdot\text{SO}\cdot\text{S}\cdot\text{CH}_2\text{CH}=\text{CH}_2$) and from *Arctium minus* (a lactone) were all inactivated by cysteine, suggested that these substances may exert their bacteriostatic effect by interference with the normal functioning of sulfhydryl groups in bacterial metabolism. This concept has since been elaborated experimentally and speculatively by Cavallito and his colleagues (105, 212 to 215), Geiger (216, 217), and Rinderknecht *et al.* (4). The list of antibiotically active agents so affected now includes: penicillic acid (216); streptomycin (214, 218); fumigatin, and various synthetic benzo- and naphthoquinones (217b); geodin (4); the unsaturated lactones anemonin, dicoumarol and parasorbic acid (219); and acrylophenone (216) and similarly constituted unsaturated ketones containing the grouping $\text{R}\cdot\text{CO}\cdot\text{C}=\text{C}-$, in which R is aryl, and a hydrogen atom is present in either the α - or β -position (4, 217b). In the case of the quinones, unsaturated lactones (213) and α,β -unsaturated ketones (216) the reaction with cysteine or other thiols is known or presumed to involve the addition of the sulfhydryl group to the double bond, and in general is irreversible (an exception is dibenzoyl-ethylene (217b), while with gliotoxin (105), streptomycin (218), and pyocyanine (212) it can be reversed by oxidizing agents. Some antibiotics (penicillin, streptomycin) are inactivated at an appreciable rate only by compounds containing basic groups in addition to the sulfhydryl group (cysteine, glutathione, etc.) (214). These and other observations pertaining to the differential reactivity of antibiotics towards sulfhydryl compounds (214), amplified by the finding that the bacteriostasis induced by gliotoxin and the thiosulfinate mentioned above (but not that by penicillin and streptomycin) can be reversed by the subsequent addition of cysteine (215), form the basis for speculations as to the varying modes by which antibiotics may interact with sulfhydryl-containing bacterial enzyme systems. A more specific avenue of approach to the problem is provided by the interesting discovery of Geiger (217a) that in the quinone group only the (weak) bacteriostatic activity against gram negative organisms is abolished by reaction with sulfhydryl while that against gram positive organisms is not, or not greatly, affected. Among the unsaturated ketones studied only dibenzoyl-ethylene showed this behavior, but contrary to expectations it was

found that a specific affinity of this compound for succinic dehydrogenase, a sulfhydryl-containing enzyme occurring also in certain bacteria, is not involved (217b). Employing Cavallito's hypothesis one would then have to suppose that the action of quinones on gram positive organisms is not exerted via sulfhydryl-enzyme systems, while with most unsaturated ketones it is so mediated. Basing their argument mainly on the much greater effectiveness towards gram positive than towards gram negative bacteria shown by the ketones of the type specified above, Rinderknecht *et al.* (4) tentatively conclude that the responsive sulfhydryl-containing enzyme system is different in the two classes of microorganism, or that such enzymes in the gram negative organisms are less accessible to these compounds. In general it would seem that with the experimental approaches hitherto used the realm of speculation is soon reached, and that further fruitful expansion of the sulfhydryl hypothesis will require a study of the interaction of antibiotics with specific bacterial enzyme systems possessing sulfhydryl groups obligatory for their action.

In the case of aspergillic acid (which is not inactivated by cysteine) the antibacterial effect may be due to withdrawal from the metabolic environment of the bacterial cell of heavy metals, particularly iron, for which this compound has a marked affinity (220). Such a mechanism is known to be operative with certain synthetic chelating compounds possessing bacteriostatic activity (221).

Gramicidin (184, 225), tyrocidine (226), and subtilin (225) (and presumably other polypeptide antibiotics of predominantly basic character) are highly surface active, and their antibacterial effectiveness *in vitro* probably depends to a large extent on this property. Also the fatty acids and lipids mentioned on p. 564 undoubtedly exert their effect in this manner. Correlatives of this mode of action are the lack of activity of most of these substances in the presence of serum and their cytotoxic and hemolytic effects. The nature of the bactericidal action of surface-active agents in general has been discussed by Valko (222), Hotchkiss (223), and Glassman (224).

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INSECT BIOCHEMISTRY

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Insect biochemistry has not been reviewed in these pages since 1940 (1). The three departments of the subject in which the most notable advances have been made since that time concern nutrition, which has been fully reviewed by Trager (2, 3), pigment metabolism, and the chemistry of the cuticle.

PIGMENT METABOLISM

Much, indeed almost everything, remains to be done on the straight chemistry of insect pigments; but it is already evident that the study of pigment metabolism may lead to results of the greatest biological interest. Pigments appear as the end products of important metabolic processes, and their occurrence may be influenced by subtle changes in metabolism associated with alterations in habit or environment. Pigment chemistry may illuminate phylogeny and systematics, and the biochemistry of pigment formation throws light upon the action of genes. The following survey of recent work is intended to illustrate some of these points. But it is well to stress at the outset that large sections of the chemistry that will be mentioned rest upon very insecure foundations. Useful reviews have been published by Timon-David (4, 5).

The most important insect pigment is that loosely referred to as melanin. The amount of melanin produced is a character of certain mutant forms, for example in *Tribolium* (6). It has been found by Danneel (7) that there is no difference in tyrosinase content between the wild type, ebony, and yellow races of *Drosophila*. Apparently the genes $+$ (brown), y (yellow), e , and b (black) have nothing to do with the production of chromo-oxidases, but influence some other component in the pigment-forming process. In the tyrosinase complex there are at least three components: monophenolase (which may perhaps be diphenolase plus an α -quinone), which converts tyrosine to dihydroxyphenylalanine;¹ diphenolase, which is a copper protein compound, cyanide sensitive, and bound to the cell structure, and which converts "dopa" to the red substance hallachrome; and enzyme III which converts

¹ Commonly known as "dopa."

hallachrome to a colourless substance and then to melanin. In a more recent paper Danneel (8) demonstrates the existence in fly extracts of a dehydrogenase which has exactly the same properties as enzyme III in being destroyed at 60°C., not bound to cell structure, and not inhibited by cyanide. He therefore suggests that enzyme III and this dehydrogenase may be identical and that the red quinone of dihydroindol-carboxylic acid (hallachrome) is not converted into dioxyindol-carboxylic acid by hydrogen transfer, as Raper supposed (114), but is converted into the quinone of dioxyindol-carboxylic acid (which is likewise red) by dehydrogenation, and this is then transformed by unknown stages into melanin. In the egg of *Melanoplus* tyrosinase exists in the form of protyrosinase, which is activated by various surface-active substances, perhaps as the result of exposure of certain groups through a mild denaturation and resultant opening of bonds (9, 10).

From the point of view of general biology the study of the eye pigments of *Drosophila*, *Ephestia*, and other insects has been particularly instructive. The wild type *Drosophila* eye owes its colour to the presence of two primary pigment components: a red water-soluble and a brown water-insoluble fraction. The red pigment is yellow in acid, red in alkaline solution. In alcoholic extracts it is almost wholly in the oxidised state but can be readily reduced to a colourless form. The brown pigment is red when reduced, yellow when oxidised (11). Ephrussi & Herold (12) describe methods of extraction and quantitative estimation of these pigments. They are the end products of two largely independent chains of reaction, but gene mutations at certain loci can affect both components simultaneously. For example, the gene *st* almost entirely suppresses the formation of the brown pigment without interfering with the formation of the red, while the gene *bw* totally suppresses the formation of the red pigment, but somewhat decreases the amount of brown.

The chemical basis of this gene action has been most fully worked out in the case of vermilion. In the presence of the normal allele of the vermilion gene, a so-called v^+ substance is formed in most, if not all, of the tissue cells (13) and liberated in the blood. As the result of a series of observations, fully reviewed by Beadle & Tatum (14) and by Ephrussi (15), it has been established that the v^+ substance is kynurenine, derived from tryptophane. Crystalline

kynurenine sulphate will lead to normal pigmentation if injected into vermilion-brown *Drosophila* larvae. Kynurenine can be detected in the body fluid of *Drosophila* and in the eggs and pupae of *Bombyx*; it is used up as the eye pigments or serosal pigments are developed (16). It is presumably derived from the oxidation of α -hydroxytryptophane which is equally effective when injected into vermilion-brown larvae (17, 18).

Synthetic (racemic) kynurenine is only half as active as natural L-kynurenine (19). The amount of eye pigment produced in an *Ephestia* of *aa* strain (which likewise is deficient in the brown eye colour) is directly proportional to the quantity of kynurenine injected into the pupa (20); and if it is injected into a female *Ephestia* of this genetic constitution, some is transferred to the oocytes so that the young larva on hatching from the egg shows a normal pigmentation of the eyes (21).

Tryptophane, α -hydroxytryptophane, and kynurenine represent successive steps in the formation of the brown pigment. Each of the stages is probably an oxidation reaction (22). It has therefore been supposed that a^+ and v^+ genes intervene in tryptophane metabolism by producing specific oxidising enzymes. In the absence of this genic activity a greater absolute amount of tryptophane persists in the body of the adult *Ephestia* (23). The amino acid is in fact incorporated in the proteins throughout the tissues, so that this single mutant gene modifies profoundly the composition of the proteins of all the cells (24). More recently it has been found, however, that homogenised tissues of *Ephestia*, both a^+a^+ and *aa* equally, can oxidise tryptophane with the production of the brown pigment. Presumably the action of the oxidising enzyme is suppressed or inhibited in the *aa* mutant (25). That would be comparable with the state of affairs in the blood of insects, where the interaction of tyrosine and tyrosinase is inhibited by the presence of a dehydrogenase (p. 608) (96). The same phenomenon is seen in the race of *Calliphora erythrocephala* with white-eyed females. The substrate and enzymes necessary for pigment formation are present in this race, as is shown by the fact that pigment will develop at very low temperatures; its formation under normal conditions seems to be inhibited in some way (26). In the white-eyed mutant of *Ephestia*, also, the gene *Wa* inhibits not the production of kynurenine but the formation of pigment from this precursor,

apparently by influencing the quantity or the nature of the carrier protein with which the pigment is normally associated (27, 28).

The next step in this pigment-forming process is the conversion of kynurenine into the unknown cn^+ substance, believed by Kikawa (16) to be the compound derived from kynurenine which gives a positive diazoreaction. For the production of this substance, which seems to be the chromogen or immediate precursor of the pigment, the cn^+ gene is necessary. In the absence of this gene, kynurenine suffers oxidative deamination and ring closure to give kynurenic acid, an end product devoid of chromogenic properties (19).

The chemistry of the final pigments is far from being understood. There is evidence that the red pigment of *Drosophila* is multiple in nature (29). By chromatographic fractionation no less than 5 components have been recognised (30). The brown pigment in *Drosophila* appears to be a single substance; but the pigments of *Drosophila* and *Ephestia*, although derived from the same chromogen, are chemically distinct; and in *Ephestia* the epidermal pigment differs from the eye pigment, although both are dependent on the $v-cn$ system (31) and both are affected by the injection of kynurenine (21).

These groups of pigments are termed "ommochromes" ("ommines" and "ommatines") by Becker (31). In *Ephestia* the chief ommochrome is the red compound, soluble in ammonia and formic acid, termed "skotommine." There is also a yellow component (confined to the corneal pigment cells) (28) which is soluble in water and is separable into a dialysable fraction and an undialysable fraction termed "xanthommine" (32). The brown pigment in the eyes of Diptera is regarded as an ommatine of smaller molecular weight. The formation of all these is influenced by the gene-controlled production of kynurenic acid.

Little is known of the constitution of these substances. The redox behaviour of the ommatines, with reduction to a more deeply coloured material and ready autoxidation, may be a reversible change from quinone to semiquinone. They appear to be amphoteric substances with free amino and carboxyl groups. The small molecule chromogens which are first formed combine with a "carrier system" and the later stages of pigment formation go forward in this combination. During the later stages the pigment-

forming reactions seemingly can branch along two paths, one leading to the ommatine type the other to the ommine type (skotommine). It is along these lines that Becker seeks to explain the different end products of the action of a single gene (31, 33). In this connection it may be noted that the introduction of 1 μ g. of kynurenine in an *Ephesia* of *aa* constitution leads to the formation of about double that quantity of eye pigment. The pigment in fact is formed by the union of kynurenine with other substances (20).

The diverse pigments that may arise in the course of metabolism from a single substance with a coloured prosthetic group may be illustrated by the fate of haemoglobin ingested by blood-sucking insects (34). In *Rhodnius prolixus* a little haemoglobin is absorbed without being digested. It is denatured in the body fluid to produce parahaematin. Some of this is taken up by the salivary glands and converted to a cherry red pigment with properties resembling haemalbumin. Some is taken up by the pericardial cells and converted to biliverdin or, experimentally, after large injections of glucose, to bilirubin. Some is absorbed by the cells of the gut wall and there transformed into (a) an altered haematin giving a pyridine haemochromogen different from pyridine protohaemochromogen; (b) a corresponding verdohaem pigment of the choleglobin type; (c) biliverdin, which is ultimately discharged into the lumen of the gut; and (d) free iron which accumulates in the cells throughout the life of the insect. Some parahaematin is taken up from the body fluid by the follicular cells of the ovary and deposited in the yolk of the eggs, giving these a pink tinge. It remains unchanged during embryonic development so that the residue of the yolk gives a bright red colour to the gut contents of the newly hatched bug. Only then is it digested and the normal end product, the iron porphyrin, set free. After injecting haemoglobin into the body cavity of *Rhodnius* all these processes are exaggerated and much of the biliverdin produced is discharged by the malpighian tubes which may be filled with the blue-green droplets. In some blood-sucking insects no haemoglobin appears to pass the gut wall; in others the course of breakdown differs markedly from that in *Rhodnius*. In *Pediculus*, for example, it appears to be unchanged haemoglobin that is absorbed and deposited in the eggs. But there is no evidence that any of these pigments is other than

an accident of metabolism without any physiological function.

The functional haemoglobin in the blood of the larva of *Chironomus* is broken down throughout larval life and gives rise to inclusions of bilirubin and biliverdin in the fat body (35). These deposits are lacking from certain individual larvae which have no haemoglobin. It is the accumulated biliverdin which is responsible for the green colour of the newly emerged fly. Biliverdin collects also in the cells of the gut wall; it is discharged into the lumen during the pupal period and voided with the excreta by the adult. Some of the haemoglobin is also discharged as such into the gut lumen and there converted into biliverdin [as indeed may happen in *Pediculus* (34)].

The question arises whether the haemoglobin that has been reported in various other insects is of functional significance. Recent work on Chironomid larvae suggests that such haemoglobin as they contain is of doubtful use in many species, even under conditions of reduced oxygen tension (36, 37), although it does function as an oxygen carrier under special circumstances in some species. The haemoglobin which occurs in the fat body cells and some other tissues of the *Gastrophilus* larva has been studied in detail by Keilin & Wang (38).² The prosthetic group is the same as that of horse blood, but the protein is quite different. It has a molecular weight of about 34,000 (39), half that of the blood haemoglobin of vertebrates, each molecule containing only two haem nuclei. It has a high affinity for oxygen, a low affinity for carbon monoxide (about 800 times lower than horse blood haemoglobin). In this respect it stands among all the known haemoglobins at the opposite extreme to the haemoglobin of *Chironomus* (40). It is very easily oxidised to methaemoglobin. It is therefore to some extent intermediate in properties between the two groups of haematin-protein compounds: the oxygen carriers and certain oxidising catalysts. The functional significance of this pigment remains to be reported.

In the squash bug *Anasa tristis* there is a breakdown of chlorophyll which is to some extent analogous to the breakdown of hae-

² It is of historic interest to record that this study was begun by Professor Keilin 25 years ago. Its completion was delayed and the author's attention diverted into other channels by the discovery of cytochrome in the course of the investigation.

moglobin in *Rhodnius* (41). A red pigment in the form of tiny crystals in the testis, salivary glands, and epidermis appears to be a pheophorbide derived from chlorophyll. A green pigment in the fat body and pericardial cells is Gmelin positive and seems to be a tetrapyrrolic chlorophyll derivative analogous to biliverdin. The yellow pigment in the malpighian tubes appears to be identical with the yellow-green fluorescent material formed by oxidation of the green pigment. Possibly it is a chlorophyll analogue of urobilin. Alongside this breakdown of chlorophyll, increasing quantities of free magnesium accumulate in the gut and malpighian tubes. The carotenoids seem not to be absorbed, so that the contents of the third ventricle are orange in colour.

It has come to be realised that the colours of insects are commonly due to complex mixtures of pigments and that the same outward appearance may be produced by very different means. The green coloration of insects is a striking example of this. Junge (42) confirms the earlier view of Przibram & Lederer (115) that in all cases the green colour is due to a mixture of blue and yellow components in varying proportions. The blue-green pigment in *Sphinx*, *Tettigonia*, *Meconema*, and *Dixippus* is a bile pigment-chromoprotein which gives a positive Gmelin reaction. The prosthetic group differs from biliverdin and from the pterobilin of Wieland & Tartter (43) which is an isomer of biliverdin. The yellow pigment is also a chromoprotein of which the prosthetic group is said to be xanthophyll in *Tettigonia*, *Meconema*, and *Sphinx*, a carotene in *Dixippus*. The protein seems in some cases to be an albumin, in others a globulin. For these mixtures of carotenoid and glaucobilin-containing green proteins, Junge suggests the name "insectoverdins." Mixed with them in the epidermis there may be some free carotenoid.

Okay (44) does not wholly support these findings. In both predaceous and phytophagous Orthoptera he claims that the small amount of carotene-albumin present does not influence the green colour. The chief yellow pigment is water-soluble and not a chromoprotein. In the Pentatomid *Nezara* the compounds are totally different. The yellow component consists largely of granules of xanthopterin, while the blue component is a diffuse pigment resembling the anthocyanins of plants. In *Chrysopa perla* the yellow pigment is a carotene-albumin, the blue is neither a bile pigment

protein nor an anthocyanin. Finally it may be recalled that the Pieridae *Colias*, *Euchloë*, etc. achieve their green wing markings after the manner of the impressionist painters, by the juxtaposition of black (melanin) and yellow (xanthopterin) scales (46).

The protein carriers clearly play an important part in the properties of many of these pigments. The predominant pigments in the hind wings of grasshoppers of the genus *Oedipoda* are chromoproteins consisting of a carotenoid combined with a protein. In the blue *O. coerulea*, the red *O. miniata*, and the yellow *O. aurea* the free carotenoid is brick red and appears very similar to, if not identical, with astaxanthin. The colour seems to be determined by the protein with which the same carotenoid is combined (45). In the locust *Schistocerca* there is a pink, mauve, or light blue tint at the base of the hind wings. The development of this colour seems to be dependent on exposure to sunlight (47).

There is little agreement about the nature of the common yellow pigments of the locusts and grasshoppers. Grayson & Tauber (48), who studied extracts of whole insects minus the alimentary canal, consider that carotene with a trace of xanthophyll is responsible for most of the yellow colour in *Melanoplus*, not only in the fat body but, in the light phase forms, in the epidermis. Chauvin (49) recognizes at least three main components in *Locusta*: (a) a yellow-green pigment with a yellow-green fluorescence in ultraviolet light, perhaps a flavone. (b) A brown pigment in the form of granules in the epidermis, termed "acridioxanthin." This was regarded by Becker (50) as an ommatin, as defined by him; but Chauvin (51) claims that it differs in properties from the ommatins in some important particulars. He regards it as an anthocyanoside, but real chemical evidence is still lacking. This brown pigment is turned bright red by acids and is excreted in this form by the malpighian tubes of the adult insect. (c) Carotenoids consisting of (i) a mixture of α - and β -carotene which colour the epidermis yellow and occur also in the fat body and (ii) xanthophyll, confined to the epidermis. Recently, Goodwin & Srisukh (52) have demonstrated the multiple nature of the yellow pigments in the epidermis of *Locusta* and *Schistocerca*. Besides the so-called acridioxanthin of Chauvin, astaxanthin identical with that of the lobster, and β -carotene also contribute. At sexual maturity, β -carotene, always present in the fat body, begins to accumulate

in the epidermis and astaxanthin to disappear, an observation which illustrates once more the relation which seems to exist between carotenoid metabolism and sexual function. Similar carotenoids occur in *Leptinotarsa* (53).

The relative quantities of these various pigments, including melanin, may be influenced by factors of many kinds. It is well known that locusts reared in crowds acquire a "gregarious" coloration in which dark and orange colouring predominates. This same result of crowding and agitation occurs in certain caterpillars, *Laphygma exigua* (54) and *L. exempta* (55); those reared in a crowd are dark and velvety. The gregarious forms are more active; presumably it is the increased activity which influences pigment metabolism in some way. The same effects can be produced in these caterpillars by keeping them in the dark. Sexual differences in coloration are a common phenomenon in insects. In the case of certain Odonata the sexual differences are considered by Becker (56) to be different stages in a process of reversible oxidation-reduction of a single pigment. For example, in the dragon fly *Sympetrum* the females are yellowish brown with the ommatin in the oxidised state; the males are red with the pigment in the reduced state. Becker even suggests that this may be a manifestation of some general metabolic difference between the sexes. Thus, in the Pierid *Gonepteryx rhamni* leucopterin predominates in the pale yellow wings of the female, leucopterin being an oxidation product of xanthopterin which predominates in the sulphur-yellow wings of the male. The Psyllid *Psylla mali* owes its usual green colour to a bright green pigment which is produced when the large bacilli carried in the mycetome are grown on a medium containing apple juice. *P. mali* infesting a cherry tree was found to be brownish red, and if the bacillus was grown on a medium containing cherry extract it produced a red pigment (57). Similarly, the symbiotic bacteria of *Aphrophora alni* produce the ochreous yellow pigment of their host, and the corresponding bacteria in *A. salicis* give rise to the red-brown colour of this insect (58).

In his classic work on the pigments of the Pieridae Hopkins described these coloured substances as "waste products which function in ornament." Hopkins, with the methods available at that time, satisfied himself that they were derivatives of the purines, the white substance being uric acid itself. It is only in quite

recent years that they have been shown to belong to the pteridine ring system, the nucleus of which was in fact synthesised by Kühling at about the same time as Hopkins' work. The recent advances in the chemistry of the group are fully reviewed by Gates (59) and need not be recapitulated here.

Biochemical interest in the pterins has been greatly increased by the wide distribution which they have been found to enjoy and by the many possible links which they may have in metabolism. Are they, after all, connected in some way with purine metabolism? The occurrence of small quantities of uric acid and hypoxanthine (60), xanthine (61), isoguanine (62), and allantoin (63) along with leucopterin and xanthopterin in the wings of Pierids suggests that that may be so, although uric acid in the excreta seems never to be accompanied by pterins (64). On the other hand, from the standpoint of structural chemistry it is plausible to regard the pterin ring as a derivative of the riboflavin molecule. (The parallel occurrence of riboflavin and xanthopterin in man has been emphasised by Koshara.) Now in many insects riboflavin accumulates in unusually large quantities in the malpighian tubes (65, 66); so that there exists a possible connection with the flavin-protein system of cellular respiration and the metabolism of vitamin B₂. Indeed Bodine & Fitzgerald (67, 68, 69) claim explicitly (though the chemical evidence is not yet complete) that in the egg of *Melanoplus* riboflavin is converted into pterin. Interest in the pterins has been further increased by the discovery that "folic acid," which is an essential food factor for insects (70, 71, 72), contains the pterin nucleus in the form of pteroylglutamic acid. The application of the method of paper chromatography to the analysis of mixtures of pterins, as described by Good & Johnson (73), should facilitate the study of pterin metabolism in insects.

The bearing of pigment chemistry on systematics has been emphasised in a series of papers by Ford (74, 75, 76). The pterin pigments are less closely restricted to the Pieridae than Hopkins supposed. They probably occur in many Lepidoptera and in other orders. Anthoxanthins are widely spread among Lepidoptera; they are always derived from the food, but the ability to absorb and deposit them in the wings is capricious. For example, the Saturnine butterflies *Pararge egeria* and *Coenonympha pamphilus* both feed on the same grasses, but only the latter deposits an-

thoxanthins in the wings. But even so the distribution of anthoxanthins may bear some relation to systematics. They are very rare in Pieridae, but they are found in the aberrant South American family Dismorphiinae and they occur also in all three Palaearctic "wood whites" (*Lepidea*) which have been united with the Dismorphiinae on structural grounds. Among Papilionidae anthoxanthins are found throughout *Parnassius* and in almost all species of *Graphium*. The types of red pigment occurring in the genus *Delias* are likewise tied to particular subgeneric groups (75) and the same applies to the red pigments in the Papilionidae. Here it is interesting to note that the red pigment of *Papilio hector*, *P. aristolochiae*, and their allies (Type A) is different from the red pigment of the forms of *P. polytes* which mimic them (Type B). This seems to rule out the idea of the same gene mutation evoking the same set of characters in model & mimic (76), although, as we saw above, (p. 598), the homologous genes *a* in *Ephesia* and *v* in *Drosophila* can lead to the production of pigments that are chemically different even in different parts of the same insect.

THE CUTICLE AND EGG SHELL

Chitin is commonly regarded as the most characteristic component of the insect cuticle, but Richards (77) is inclined to consider protein to be the fundamental constituent because in some butterfly scales it has proved impossible to demonstrate chitin by the chitosan test. When the scales of *Ephesia* are first laid down chitin is absent; it is incorporated progressively as they mature (78). Chitin in the cuticle ranges from 25 per cent to 55 per cent of the dry weight, with an average of 33 per cent (79). It is always closely associated, perhaps chemically combined, with protein (80). Stacey (81) and Haworth (82) class chitin in the loose sense among the mucopolysaccharides. It is interesting in this connection to recall that insects do not secrete mucus; they protect their delicate gut wall with a peritrophic membrane of chitin and protein. Perhaps this may be regarded as a variant on a common biochemical theme.

The bulk of the nonchitinous material of the cuticle is protein, which varies in amount from 25 to 37 per cent (79). The extractable protein, named "arthropodin" by Fraenkel & Rudall (83), has some peculiar characters. It is highly soluble in hot water and

after precipitation in 10 per cent trichloroacetic acid it redissolves on heating (84). In the cuticle of *Sarcophaga* and *Sphinx* larvae (80) there are two proteins present: (a) Arthropodin, water-soluble and containing no carbohydrate, resembling sericin in general properties, though the serine and glycine contents are low, tyrosine and tryptophane being present; (b) a second protein, present in small amounts only, which is extractable with 5 per cent sodium hydroxide and contains a considerable amount of carbohydrate. Neither protein contains sulphur.

The crystalline structure of the chitin micellae has been defined in detail by Meyer & Pankow (85). Fraenkel & Rudall (83) suggest that within the natural micellae chitin is bound to protein. The cuticular protein, arthropodin, exists in the fully extended β -configuration. In this form the protein chains may be expected to fit much better with the chitin chains; for three amino acid residues will give a periodicity of 10.2 \AA (i.e., 3.4×3) which agrees well with the length of the chitobiose unit (10.4 \AA). These authors suggest that a part of the x-ray diffraction picture obtained with fresh cuticle may be due to protein. The chitin-protein ratio is commonly around 55:45. The cuticle may consist of alternating monolayers of protein and chitin; it may even be that the monolayer of β -protein synthesises the new chitin layer, the two structures forming interpenetrating lattices (83). As seen with the electron microscope there appear to be differing molecular densities among the micellae in successive layers of the cuticle (86). After purification with alkali some chitinous membranes give microfibrils of chitin which after drying have a diameter ranging from less than 100 \AA to about 300 \AA . Perhaps these values represent the diameters of the chitin micellae (87).

In the endocuticle of the *Calliphora* larva the crystallites show an entirely random orientation in planes parallel to the surface, but they can be orientated in any desired direction by compression or extension of the cuticle. Thus when the larva rounds up before forming the puparium there is a 12 per cent increase in its circumference, and the consequent stretching makes the crystallites lie in the transverse axis; this in turn is reflected in the tendency for the fully formed puparium to split in this direction (84). In hairs and bristles the micellae are orientated in the long axis. In the bristles of *Drosophila* they are more highly orientated in the ridges than in the intervening material. The growth and form of these

bristles is largely influenced by the fine structure and properties of the substance of which they are composed. Perhaps the bristle may be regarded as an object blown in plastic material—a long-chain high polymer in the rubber-like state, almost devoid of cross linkages between the chains. As the substance of the wall ages it hardens. In the formation of a normal bristle there must be a nice balance between changes in content and changes in the wall. The various mutant bristle types in *Drosophila* may be explained by upsets in these orderly processes or by abnormalities in the biochemical properties of the wall (88). Iridescent colours in insect scales and cuticle are due to periodic structures which probably arise spontaneously by crystallization within the substance of the cuticle. The puparium of blowflies, for example, when viewed from within, shows interference colours of this type (89). In the chitinous cocoon of the beetle *Donacia* there is a spontaneous separation into laminae in which the micellae assume two preferred orientations (90).

The hardest parts of the cuticle contain least chitin. The cuticle of the *Calliphora* larva has 54.8 per cent of chitin, that of the puparium 32 per cent (79). The added substance is protein: the total nitrogen in the cuticle rises from 9.78 per cent to 11 per cent as the percentage of chitin falls (79). As hardening proceeds the mobility of the crystallites is lost (84) and the intermicellar spaces become filled with amber or brown material with a refractive index approximating to that of chitin so that the "form birefringence" of the cuticle disappears (91).

This insoluble impregnating material is tanned protein, termed "sclerotin" by Pryor (92). In the ootheca of the cockroach it exists in the absence of chitin. Here a watery solution of protein is secreted by the left colleterial gland. The right gland secretes a solution of protocatechuic acid (93). On mixing these secretions the protein becomes hard and dark; polyphenoloxidase present in the secretion oxidises the protocatechuic acid to the corresponding quinone which tans the protein. Likewise in the hardening of the cuticle at pupation, dihydroxyphenols are added to the chito-protein membrane (91). Various phenolic substances seem to be concerned: protocatechuic acid in the *Calliphora* puparium, dihydroxyphenyl acetic acid and dihydroxyphenyl lactic acid in the adult *Tenebrio*. They are presumably derived from tyrosine by way of "dopa" (94).

In the puparium of *Calliphora* and *Sarcophaga* at the time of hardening, tyrosine and tryptophane accumulate in the outer endocuticle (the part which later becomes sclerotized) (95). The increase in weight of the cuticle at this time (8.8 per cent) agrees with the fall in the tyrosine content of the blood [9.1 per cent of the cuticle weight (83)], and during hardening there is a large decrease in the total tyrosine of the larva (80). Orthodihydroxyphenol, presumably derived from tyrosine, accumulates in the outer parts of the cuticle just when hardening begins. Polyphenol-oxidase is concentrated in the epicuticle. Here the polyphenol is presumably oxidized to orthoquinone which diffuses inwards through the cuticle and this becomes tanned and hardened from without inwards. The absence of quinone from the inner layers of the cuticle in these blowflies results from the presence of active reducing substances (96). It has been suggested (though without clear-cut experimental backing) that local differences in hardness of the cuticle may result from differences in the entry of catalase which diminishes the activity of the peroxidase (97).

The darkening of the cuticle is largely due to the tanned protein (91); but some undeaminated tyrosine is probably utilized for melanin formation (80, 83). In *Drosophila*, for example, the darkening associated with tanning and the blackening due to the deposition of melanin seem to be differently influenced by different genes (98). In the full grown larva of *Sarcophaga* tyrosine and tyrosinase are present together in the blood, but blackening does not take place *in vivo*. According to Dennell (96, 99) this is because the oxidation of tyrosine is inhibited by the low oxidation-reduction potential in the blood; and this in turn is ascribed to the action of a dehydrogenase acting upon glucose as hydrogen donator. There is a steady fall in the oxidation-reduction potential as tyrosine and tyrosinase appear in the blood; an abrupt rise coincides with the initiation of puparium formation. Tyrosinase activity is thus released, dihydroxyphenol (probably "dopa") is produced, and hardening and darkening can proceed.

The tanning of the cuticle, with the consequent "drying" and condensation of the protein, provides some degree of impermeability to water. Very dark cuticles tend to be impermeable (100); the hard pronotum of *Lucanus* and other beetles is still hydrophobic and impermeable after the outer parts have been ground away

(79). But the most important component in the waterproofing of cuticle is epicuticle, a complex structure in which several layers can be recognised (86, 95). These have been studied in detail in *Rhodnius* (101) and in the adult *Tenebrio* (102). The first to be deposited is the innermost or "cuticulin" layer, believed to consist of lipoprotein which is perhaps denatured and condensed and is finally tanned along with the other proteins of the outer layers. A viscid fluid containing material which reduces ammoniacal silver hydroxide (perhaps dihydroxyphenol associated with protein) is discharged on the surface of the cuticulin, and then a layer of wax is laid down over this. The wax has an average thickness of about 0.25μ ; it varies in character from a soft grease to a hard white crystalline substance. In the pupal wax of *Pieris* there are yellow and white fractions with different properties (103). If these waxes are extracted from the cuticle with chloroform and spread as a thin film on some artificial membrane they effectively prevent the transpiration of water. On warming this system a "transition" temperature is reached, at some 5° to 10°C . below the melting point of the wax, at which the transpiration of water increases abruptly. This temperature varies from 30° to 60°C . with the waxes from different insects. The values agree well with those obtained for the sudden rise in the rate of transpiration from the intact insect of the same species (103, 104). The innermost layer of wax is probably the most effective in restricting the passage of water (105). It seems likely that the surface of the substrate membrane on which the wax is deposited influences the orientation of the innermost wax molecules (103).

The mechanism of secretion of these long wax molecules is obscure. Lees & Beament (106) have studied the secretion of the wax with which the female tick *Ornithodoros* waterproofs its eggs. This wax appears to be solubilized with protein which is detached from the wax and perhaps reabsorbed as secretion is completed.

The wax layer of the cuticle is protected by a further layer, termed the "cement layer," poured out from the unicellular dermal glands. This substance reduces ammoniacal silver very actively, but only after extraction in boiling chloroform. Perhaps it too consists of phenol-tanned protein associated with lipids (102).

There is some evidence that the lipoproteins which form the cuticulin layer are produced by the oenocytes (101, 102). The

deposition of the chitin and protein layers coincides with the transfer of the reserves of glycogen and protein from the fat body to the epidermal cells (101).

The egg shell.—Recent work by Beament (107, 108) has shown that the egg shell has much in common, fundamentally, with the cuticle, but is even more complex. The chorion of the egg in *Rhodnius* consists of seven distinct layers, together with a secretion of "cement" added to the outer surface when the egg is laid. All these layers are freely permeable to water; waterproofing is provided by a thin wax layer of about 0.25μ secreted by the oocyte on the inside of the shell. Thus, in the fully formed shell, outside this wax layer, there is (a) an inner "polyphenol layer" of discontinuous tanned granules; (b) a resistant protein layer of tanned protein 1 to 2μ thick, containing diffuse polyphenol; (c) an outer polyphenol layer consisting of smaller granules; (d) an "amber layer" of tanned protein to which oil is added after secretion; (e) a laminated layer some 8μ thick consisting of soft protein containing polyphenols; and (f) and (g) the layers of the exochorion, which appear to consist of lipoprotein secreted preformed and thus resembling the "cuticulin" of the cuticle. The inner layer of the exochorion is soft, the outer thin and more resistant. Here, as in the cuticle, the proteins are modified by association with lipids and by phenolic tanning. It is possible that in the resistant layer (b) the protein may also be polymerised or "vulcanised" through sulphur linkages.

In the egg of *Lucilia*, two distinct protein layers with different properties compose the chorion, and both of them, together with the thick chorionic vitelline membrane below, have bound lipid material incorporated with them before the egg is laid. The egg is rendered comparatively waterproof by means of a thin wax layer laid down by the oocyte between the chorion and the chorionic vitelline membrane (109). There is some evidence of an oily layer between the exo- and endochorion in the egg of *Culex* (110). The behaviour of the *Culex* egg illustrates the remarkable mechanical effects which result from surface tension in combination with the varying wetting properties of the different parts of the surface of the shell.

In the egg of *Melanoplus* there is a "hydropyle" at the posterior extremity whose underlying cells are responsible for the uptake of water by the developing egg. During diapause these cells appear

to produce a waxy secretion by which the hydropyle is occluded. Slifer (111, 112) has recently shown that if the chorion of the egg is first removed, by immersion in sodium hypochlorite, diapause may be terminated by subsequent immersion in xylol and other wax solvents. Water can then pass into the egg and development is resumed. In the chorion of the stick insect *Bacillus* there is about 38.4 per cent of ash at the beginning of development. This is reduced to about 30.8 per cent at hatching, chiefly by solution and absorption of calcium carbonate which is transferred to the malpighian tubes of the embryo (113).

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NITROGENOUS CONSTITUENTS OF PLANTS

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The last review of this subject by Stewart & Street (1) in 1947 was very comprehensive, and the present reviewer had to make a choice between preparing an addendum to that review or discussing some phases of the subject that have not been covered in earlier papers bearing this title. The latter course has been chosen even though it means omitting reference to some valuable papers published in the last two years. Earlier reviews have tended strongly towards a physiological approach to the question under discussion and have stressed the metabolism of the nitrogenous constituents quite as much as, or more than, their chemistry. The same approach was used by Chibnall (2).

On the other hand, Vickery (3) stated that he presented his discussion of plant proteins from the point of view of a protein chemist interested in proteins as chemical substances. He expressed the hope that when sufficient was known about the proteins it would be possible to give adequate chemical and physical characterizations of these substances. The present review follows Vickery's approach and special emphasis is placed on those phases of work on plant proteins not covered in his review, particularly the work dealing with physical properties. Vickery gives his interpretation of what is meant by the term "plant protein" [(3), 351-52], and his views, with a few slight modifications, so closely represent the present writer's opinions that there is no need to repeat them here. Insofar as possible, however, the term "plant protein" as used here is meant in its specific, rather than its general, sense as outlined by Vickery. Nevertheless, it is often impossible to be precise in discussing the plant proteins, particularly when dealing with chemical composition.

The headings used are meant as guides, rather than as absolute divisions of the topic.

IDENTITY AND PHYSICAL PROPERTIES OF SEED PROTEINS

While the study of seed proteins is at present in advance of the study of proteins of vegetative parts of the plant, it is still far behind the study of many animal proteins. Seed proteins are

usually imbedded in a matrix of nonprotein material from which they are difficult to extract. Solubility is usually low, and drastic measures are required to separate, "purify," and dissolve these proteins. The proteins of wheat have been most extensively studied but there is no general agreement even as to the number of proteins in the wheat grain. Emphasis is placed on the cereal proteins in the present discussion, whereas discussion of the proteins in dicotyledonous seeds is designed to supplement Vickery's review (3).

Wheat proteins have probably been most extensively investigated because the unique properties of wheat gluten permit the separation of a large proportion of the seed proteins from the non-nitrogenous constituents of the grain (4). As a result, one of the major problems in studying plant proteins is overcome. The ease of this is unmatched with any other plant protein. In general, it may be said that the bulk of the proteins of wheat appear to form a complex component protein system (5 to 10). This concept, as contrasted to that of Osborne (11), began to emerge about 1930. Both chemical and physical methods of study gradually contributed to the knowledge of these proteins. Furthermore, so far as can be determined, the proteins of other cereal seeds appear to be of much the same general type (12 to 17).

Before discussing the newer results obtained in the study of wheat gluten and of comparable proteins from other cereals, it should be recognized that in all such seeds there is a "salt-soluble" fraction that differs radically from the main protein constituents. In wheat this fraction is best termed simply "nongluten," because the amount extracted varies widely with the salt solution used to extract it (18). This fraction usually occurs in small quantity, is mixed with nonprotein nitrogen and soluble carbohydrates, and is extremely difficult to study critically. Nevertheless, it has the characteristics of albumins and globulins (5, 18, 19). It is probably not a form of storage protein at all, but is rather made up of the cellular proteins of the storage organs and perhaps of the seed embryo (3, 19). Almost no critical work has been done with these proteins.

The concept of gluten protein as a component system was first advanced by Sørensen (20) in 1930. His discussion dealt only with so-called gliadin, but McCalla & Rose (5) showed that the concept could be extended to cover gluten as a whole. There has been much discussion of this concept since that time, but the balance of evi-

dence is certainly in favor of rejecting the old idea of a clearly defined prolamine "gliadin" and a clearly defined glutelin "glutenin" as distinct proteins whose mixture yields gluten. Blish (21) found that when gluten fractions were obtained by fractional solubility rather than by fractional precipitation, the earlier conclusion of McCalla & Rose was not supported. Spencer & McCalla (7) then secured results using fractional solubility methods which agreed very well with the earlier ones and showed further that Blish's failure to get concordant results was probably associated with the denaturation of the protein during his method of preparation.

Much more precise methods have yielded experimental results that support fully these earlier conclusions. McCalla & Gralén (8), using sedimentation and diffusion methods, showed that as the solubility of gluten fractions decreased the mean molecular weight of the fractions increased. Furthermore, even the most soluble fraction, although it was completely molecularly dispersed, was not homogeneous in molecular size or weight. The molecular weight of the most soluble fraction varied at least from 39,000 to 67,000 within this one fraction. Since this fraction is all included in the classical "gliadin" one must reject the notion of this prolamine as a distinct, homogeneous protein.

The heterogeneity of protein from other cereal seeds is clearly shown by work on zein from corn, secalin from rye, and hordein from barley. Watson, Williams & Arrhenius (12), Gortner & McDonald (16), and Scallet (17) have all shown that zein is polydisperse. Andrews (14) worked with secalin and gives specific figures for the diffusion constant and for molecular weight. The methods used in calculating these values do not permit demonstrating polydispersity, but the similarity of the results to those obtained with gliadin entitles us to conclude that secalin is a similar protein. Hordein has been shown by Quensel & Svedberg (13) to be polydisperse.

Schwert, Putnam & Briggs (15) investigated the electrophoretic properties of gliadin and concluded that this protein dispersed in acetate buffers was electrophoretically heterogeneous. Laws & France (22) carried out an extensive electrophoretic study of gluten from several different varieties of wheat. No differences attributable to variety could be demonstrated, but all preparations in citric acid-disodium phosphate buffers were definitely hetero-

geneous electrophoretically. Colvin & McCalla (23) have been unable to confirm the results of these studies and believe that the electrostatic properties of the various gluten fractions dispersed in sodium salicylate solutions do not differ nearly so widely as do the other physical properties. On the other hand, Scallet (17) obtained results with zein from corn that agree more closely with those of Schwert *et al.* He concluded that zein, in alcoholic solutions, contains at least six components which constitute reversible association-dissociation systems. He was not able to separate the components by fractional precipitation but they could be separated in the Tiselius electrophoresis apparatus.

The electrophoretic behavior of gluten dispersed in sodium salicylate leads to very interesting speculations regarding the surface characteristics of the protein. It is possible that gluten fractions selectively adsorb salicylate ions, and this adsorption may eliminate any electrostatic variation that normally would be exhibited by the different fractions. Such selective adsorption of phthalate and picrate ions by proteins of silk has been demonstrated by Sookne & Harris (24). If this adsorption takes place with gluten then the surface properties of egg white proteins and of gluten must be very different because the electrophoretic behavior of egg white proteins dispersed in sodium salicylate is the same as the behavior of these proteins dispersed in the conventional phosphate buffer (23). So far, very little is known about the effects of different amino acids in determining the surface properties of proteins of widely different types.

The molecular weight of the most soluble fraction of gluten as determined by McCalla & Gralén (8) has already been referred to. Considerable spasmodic work on molecular weight of cereal proteins had been carried out over a period of years but much of it yielded results that are not too convincing when considered together with values obtained in recent studies. No attempt is made to list references to such results, but the disagreement between results for "gliadin" as presented by Arrhenius (25) and for soluble gluten as presented by McCalla & Gralén (8) illustrate the argument. The best results at present available place the minimum molecular weight of soluble cereal protein fractions at about 35,000 to 40,000 (8, 12, 14, 26). The molecular weight of the more insoluble fractions of these proteins is almost certainly much higher, but the calculation of satisfactory values is seriously complicated

by the fact that such protein "dispersed" in most solvents is in aggregated rather than molecular form (8). The same studies show that the proportion of aggregated protein in the successive fractions increases with decrease in solubility, and the mean molecular weight greatly increases. As a matter of fact, it is equally clear that the molecular weight of the molecularly dispersed protein of successive fractions also increases rapidly as solubility decreases.

Molecular shape, size, and hydration have also been studied. Kuhlmann (6), on the basis of solubility and swelling studies, concluded that gluten is made up of micelles of varying length, the most soluble being the shortest and the least soluble the longest. He represents his ideas diagrammatically, showing a considerable number of micelle lengths. Neurath (27) and Mehl, Oncley & Simha (28) obtained axial ratios of from 10.5 to 11.1 for purified gliadin, using viscosity, sedimentation, and diffusion data. Neurath also estimated the diameter of the gliadin molecule to be 18 Å. His calculations involved a molecular weight of 26,000 for the gliadin. McCalla & Gralén (8) obtained a frictional ratio of from 1.90 to 1.94 for the most soluble gluten fraction. This indicates a high axial ratio, but contrary to expectations, the frictional ratios of more insoluble fractions were progressively lower. This is taken to show that the aggregates are formed by side-to-side rather than end-to-end aggregation of molecules. These "bundles" of molecules, even though they are longer than the molecules of the more soluble fraction, will have a lower axial ratio because of the greatly increased diameter of the bundle. Barmore (10) used viscosity methods and also represents his conclusions diagrammatically. He concludes that gluten protein molecules are rodlike ellipsoids of revolution with the most soluble fraction of gliadin having a mean axial ratio of 15. A second gliadin fraction had a mean axial ratio of 26, while the value for gluten as a whole is given as 41 and for "glutenin" as 47. Colvin & McCalla (23) determined the diameter, mean axial ratio, and molecular volume of a molecularly dispersed fraction of gluten. They also conclude that the molecules in this fraction are elongated prolate ellipsoids of revolution with a mean diameter of about 25 Å, a mean axial ratio of 16.7 (which is to be regarded as a lower limit until it can be confirmed by improved methods), and a mean molecular volume of 56×10^3 cubic Å. It must be stressed that these values are all averages and that they will certainly vary with the particular fraction studied. This work

also indicates that the gluten molecule is not extensively hydrated.

It should be emphasized that the foregoing discussion has omitted reference to many comparatively recent papers dealing with the cereal proteins from what may be termed the classical solubility point of view. It is believed that the results of such studies contribute very little to the understanding of the fundamental properties of the protein complex, and therefore discussion of such results is unwarranted in this review. The type of study covered by these comments is illustrated by papers by Urión and co-workers (29, 30).

It has been known for some time that the physical properties of the gluten mass depend on more than the proteins in that complex. The importance of lipid substances has been recognized and reviewed. Much of the earlier work was directed towards a study of flour quality rather than to an attempt to determine protein properties. Thus, Brooke (31, 32) and Sullivan, Near & Foley (33) emphasize the flour quality aspects, while the work of Kozmin (34, 35) dealt with the gluten itself. It has been suggested that phosphatides (36, 37), unsaturated fatty acids (34, 35, 38, 39, 40), and oxidized fatty acids (33) are important in determining the physical properties of gluten. If flour is extracted with ethyl ether or petroleum ether before the gluten is washed from it, many of these fatty substances are removed from the flour. This shows that they were not part of the gluten complex in the dry flour. If washing is done prior to extraction, some of these fatty substances become part of the adsorption complex (39). There are other fatty substances, however, that cannot be removed by the extraction of flour. It is possible that these are already a part of the complex.

The early work carried out in the reviewer's laboratory (39, 40) appeared to establish that there was a distinct loss in solubility of gluten when the more insoluble lipids were not present in the gluten complex. Gluten could no longer be completely dispersed in sodium salicylate solutions (39) and the water-absorbing capacity of the washed gluten was markedly reduced (40). Normal gluten takes up water very rapidly when immersed in dilute, weak acids such as 0.1*N* acetic. Within 2 hr. dispersion has usually reached the point where the gluten ball can no longer be handled. With gluten from old flour, or from flour subjected to extraction by alcohol, dispersion was not complete even at the end of much longer periods.

It was confirmed by McCalla & Gralén (41) that the solubility

of gluten was greatly affected by the amounts of fatty acids and lipid substances present in the flour before the gluten was washed. The proportion of molecularly dispersed protein as determined by high-speed sedimentation under constant conditions varied from 34 to 68 per cent of the total protein. Solubility was increased by extracting the fatty acids before washing the gluten and decreased by adding linoleic acid before washing. Work with protein from very old flour of low quality showed that the protein-lipid complex found with normal gluten no longer existed. Even when such flour was ether-extracted before the gluten was washed, the solubility of the protein was much lower than with normal gluten. Thus either the protein-lipid complex that occurs under normal conditions must break down during prolonged storage of the flour or the lipids that are normally adsorbed on the protein to form this complex during washing of the gluten are no longer available. Normal gluten must therefore be considered as a protein complex with some lipid substances very closely associated with, if not an actual part of, the complex.

Tucker (42) has reported a marked effect of lipids in soft wheat flour on the solubility of gluten protein. He found a much smaller effect on gluten from hard wheat. He believes that his results suggest the importance of the concentration of the lipids on the surface of the protein particles. Olcott & Mecham (43) found that 70 per cent of total lipids was extracted from flour by ether, but only 40 per cent was extracted after the flour had been mixed with water and dried by lyophilization. After the flour was kneaded into a dough and dried, less than 10 per cent of the lipids could be extracted. At least three times the lipid normally present in flours could be bound by the doughing procedure. Most of the lipid bound was associated with the gluten and the binding appeared to be to the "glutenin" rather than to the more soluble (gliadin) fractions of the protein. Some glutenin fractions containing up to 20 per cent lipids were isolated. This association between the lipids and the less soluble protein fractions is in agreement with the results of McCalla & Gralén (41), who found no difference in the properties of the molecularly dispersed fraction regardless of the amount and condition of lipid substances. It seems quite probable that some of the difficulties encountered in working with so-called "glutenin" are due to the removal of lipid from the more insoluble fractions of gluten during the separation of gliadin by alcohol

extraction. Certainly the most recent work indicates that alcohol preparation of gliadin leaves a great deal to be desired, especially if the remainder of the protein is to be studied.

Whether other cereal proteins exhibit this intimate association of the relatively insoluble fractions with lipid materials has not, so far as the reviewer knows, been investigated.

When one turns from the cereal proteins and considers the physical properties of other seed proteins, an entirely different situation appears to exist. Most plant seeds contain more than one protein but these appear to belong to the albumins and globulins and are probably much more distinct and clearly defined than are the so-called prolamines and glutelins of cereals, although not so clearly defined as the older work would lead us to believe. Vickery (3) calls attention to most of the difficulties encountered in working with such proteins. It was, until recently, assumed that the preparation of a protein in crystalline form was a guarantee of homogeneity. This can no longer be accepted, as shown by Pirie (44), Bailey (45), and more recently by Li (46). Pedersen (47), after referring to Li's work with crystalline β -lactoglobulin, says that it is not unlikely that other "standard proteins" will be found to consist of more than one major component. The work of Johnson (48) with peanut (ground-nut) proteins also raises a very important question with respect to the assumption that two definitely different sedimenting protein species necessarily mean two distinct proteins. This work is discussed in more detail below.

The most complete study of the solubility of soybean proteins is contained in work done at Connecticut, reported in Vickery's review (3). The conclusion that the total protein of soybeans is a mixture of components will hardly be challenged. Preliminary work in the present reviewer's laboratory (49) shows that soybean proteins are more readily "dispersed" (as distinct from "dissolved") than are many other of the plant proteins. Electrophoretic diagrams showed that the protein soluble in sodium phosphate buffer at pH 8.1 and temperature of 2°C. was heterogeneous. There was no difficulty in securing solutions of 1 per cent protein concentrations and these gave excellent diagrams. No accurate determination of the percentage of the whole protein was attempted, but 80 per cent of the soybean protein was present in the first extract. It is obvious that the fraction studied in the electrophoretic apparatus was a much smaller portion than this, but it was distinctly

heterogeneous. The components did not separate so clearly as with pea proteins (see below) but more than one compound was certainly present.

The proteins in pea meal have been subjected to a more complete study (50). The work of Osborne & Campbell (51) and Osborne & Harris (52) indicated the presence of three proteins, two of them globulins, in peas. The criterion of individuality was, of course, solubility. Electrophoretic studies carried out by Wetter & McCalla (50) show that only 65 per cent of the pea protein could be dispersed in phosphate buffers, and that this 65 per cent contained at least three distinct components differing in electrostatic properties. The protein system tends to separate into further components under some conditions. It is clear that the fractional precipitation procedure does not separate the components as detected by electrophoresis, as two of these appeared in each of the separate fractions prepared by precipitation. The amount of each component in the various fractions varied with differences in procedure of preparation. Thus pea proteins, like most others studied, are mixtures of components but these cannot be satisfactorily separated by usual methods of preparation.

Vassel & Nesbitt (53) and Vassel (54) have isolated two proteins from flaxseed. The tests of homogeneity applied by them convinced them that the proteins, linin and conlinin, are homogeneous products. The methods used, however, leave much to be desired, and in view of results with other proteins, one hesitates to accept the view expressed by these investigators. The whole study of flaxseed proteins is just as much complicated by solubility problems as is that of most other plant proteins. This has been shown in work by Smith, Johnsen & Beckel (55) and Painter & Nesbitt (56). The latter found much greater extraction of ball-milled meal than of meal extracted without this step. At best, however, at least 20 per cent of the protein was unextracted.

The most fundamental work with seed proteins other than those in cereals has recently been carried out with proteins from peanuts. Fontaine and co-workers (57, 58) have supplemented earlier work [(3), p. 354] with electrophoretic studies of these proteins. They use the terminology arachin and conarachin, but show (57) that both proteins contain a fast-moving component, A. A slower-moving component, B, is found in arachin but not in conarachin. Arachin, on the other hand, contained none of the minor com-

ponents that made up about 20 per cent of conarachin. It is obvious, therefore, that these names refer here to gross preparations rather than to distinct proteins. It was also reported (57) that a small fraction (3 per cent) remaining after successive removal of the arachin and conarachin contained 2.9 per cent sulfur, a value particularly high for plant proteins. These results lend emphasis to the caution needed in interpreting extraction results. It is never safe to assume that the extracted protein is a "good sample" or is "reasonably representative" of the total protein of plant materials. It is, in fact, much safer to assume that there are differences. Fontaine *et al.* say (58) that a fraction containing 90 per cent of the total protein is not truly representative of the total protein of the peanut. They found that fractions precipitated at different pH levels differed in properties and that a fraction not precipitated at pH 6.0, but precipitated when the solution was adjusted to pH 4.5, had a quantitative electrophoretic composition markedly different from either of the other fractions described and from arachin and conarachin.

The most fundamental study of peanut proteins was carried out by Johnson (48). He studied the sedimentation of several preparations and found that the behavior varied with the method of preparation. When precipitation from the original extract was accomplished by dilution there were two sedimenting species with s_{20} values of 14.6 and 9.5. When precipitation was accomplished by saturation with ammonium sulphate, only one species appeared ($s_{20}=14.6$). When stronger ammonium sulphate was used, two further species appeared ($s_{20}=6.2$ and 4.5). No trace of the $s_{20}=9.5$ species was found in preparations involving 40 per cent ammonium sulphate. The diffusion constant of the heaviest species was determined from the sedimentation diagrams as 3.2×10^7 . Molecular weight was determined to be 396,000 and the frictional ratio, 1.4. Diffusion constants were not available for the other sedimenting species.

In the second part of the study Johnson found that the species with $s_{20}=9.5$ was derived from the dissociation of the $s_{20}=14.6$ species. This was demonstrated by diluting and acidifying, and the extent of dissociation was found to be controlled largely by the extent of dilution before acidification. Dissociation was repressed by high concentrations of ammonium sulphate, but suppression was only partial in lower concentrations of ammonium

sulphate. Thus a series of sedimentation diagrams are presented by Johnson, with the $s_{20}=9.5$ species gradually disappearing. At a salt concentration of 20 per cent only the larger parent species remained. Potassium chloride acted in a similar manner, but was less efficient. Johnson believes that the splitting is equatorial rather than meridian, and hence the dissociated molecules have an axial ratio of only half that of the parent molecule. It is possible, of course, that the two half molecules might sediment at the same rate but be dissimilar in other properties. Johnson also states that the two species with lower sedimentation rates (6.2 and 4.5) are not derived from the $s_{20}=14.6$ species, but may be proteins of a different type.

Fontaine, Pons & Irving (59) established that the naturally occurring phytic acid in meals from peanut, cottonseed, and soybean is responsible for the suppression of the solubility of the seed proteins at pH values below their isoelectric points. They found phytic acid as a major impurity in isolated proteins from seed meals, the amount varying with the methods of extraction and precipitation and the degree of purification. They consider that phytic acid is an impurity rather than an integral part of the protein molecule, but point out that its presence would influence the results of electrophoretic mobility, viscosity, and proteolytic effects on solutions of these proteins.

In summarizing the present state of our knowledge with respect to the physical properties of seed proteins, one is forced to conclude that extraction is still the key difficulty in studying these properties. In many instances very good results can be obtained if the proteins can be satisfactorily dissolved or dispersed. Usually the investigator must be satisfied to work with only part of the protein from seeds and often must carry out studies on fundamental properties under anything but good conditions. Nevertheless, our understanding of these proteins has progressed rapidly in the last few years. At present we know that most seeds contain more than one type of protein and some at least appear to contain very complex component protein systems. Definite and reliable values of molecular weight, molecular shape, and size are now available for a few protein fractions. Even though these values apply only to parts of the total protein of any seed, they form the most precise information that has yet been obtained. One may hope, with some justification, for much more rapid progress in the next few years.

AMINO ACIDS IN SEED PROTEINS

Despite the difficulties inherent in determining the constituent amino acids of seed proteins many investigators continue to make such determinations. The statement by Block & Mitchell (60) that one of the most serious difficulties in amino acid analysis stems from the preliminary operation of hydrolysis will meet with general agreement. This problem has been discussed by many investigators, but despite agreement regarding some difficulties there is sharp disagreement as to the relative importance of various sources of error and as to the value of carrying out determinations that may be in considerable error. Chibnall (61) records the amino acid content of several proteins, each acid being determined by several different methods. Very good agreement is now frequently obtained using different methods—a result that Chibnall emphasizes as essential for acceptance. Vickery (62) says that the inadequacies in analytical methods are far less serious than the difficulties in selection, preparation, and purification of the protein to be analysed. He is particularly critical of the opinion that satisfactory results can be obtained by direct hydrolysis of foodstuffs. Vickery & Clarke (63) and Mitchell & Block (64) have shown that the clash of opinions is largely a matter of the basic interest of the investigator. A nutritionist is not likely to be very much concerned with the failure of his results to satisfy the protein chemist, while the latter feels that many of the techniques used by the nutritionist are entirely inadequate even to serve the end of nutrition.

Lugg (65) has recently discussed the errors in the determination of amino acids. He lists those already mentioned and stresses that there are also unknown errors.

In a review such as this, the protein chemist's view is the more valid, since interest is primarily on securing basic information about the proteins. On the other hand, if other approaches fail, even the chemist will be aided by results of a cruder type of analysis. The gross demonstration, for example, that gliadin and zein differ widely in glutamic acid content (61) is of definite value in assessing the properties of these protein fractions.

It seems essential to stress again that the closest possible agreement between results for amino acid content obtained by different methods will give no guarantee of accuracy if the preliminary steps in preparation have already destroyed or altered part of the amino acid originally present in the protein being

studied. Despite this and all other difficulties, however, it is believed that some of the recent work deserves consideration in this review. No claim of completeness is made for the review since some of the data published primarily in the interest of nutrition are very likely to escape the plant biochemist. Furthermore, amino acid values for most gross foodstuffs are of little interest in a review under the present title.

Whether it is better to approach this question from the point of view of individual proteins or seed, or of individual amino acids, is difficult to decide. In a few instances the latter appears to be better, but it cannot be satisfactorily followed in the consideration of many papers. A compromise is therefore adopted.

The sulfur-containing amino acids have been studied extensively in recent years. Lugg & Clowes (66) studied "whole" proteins from seeds of *Trifolium subterraneum* and reported "very low" values for both methionine and cystine. Johanson & Lugg (67) studied two varieties of soybeans. The varieties did not vary significantly, and cystine values were considered "moderate," while methionine values were low. Johanson (68) determined methionine and cystine in *Vicia spp.* again reporting the results as applicable to "whole" proteins. He found the cystine content of the proteins to be much the same as in soybeans (66) but the methionine content to be lower, more like that in peas. Johanson summarized the Australian work in tabular form. Cystine nitrogen in these legume seeds varied from 0.75 to 2.07 per cent of the total nitrogen in the seed. Comparable figures for methionine are given as 0.26 to 0.88 per cent. Peas were the lowest in cystine while lupins were highest. Lupins were lowest in methionine while soybeans were highest. In contrast to some animal proteins these legume seed proteins are very low in methionine and they are also lower than zein and gliadin as reported by Chibnall (61).

Evans *et al.* (69) determined the cystine and methionine content of different varieties of peas grown on different locations and subjected to a variety of fertilizer treatments. Methionine values were somewhat higher than those discussed by Johanson (68) but they were nearly all under 1 per cent. Most of the cystine values were at least double the value given by Johanson. Location and variety did not affect cystine and methionine content, but fertilizer treatment did. Despite the fact that protein content tended to be reduced, cystine and methionine were both significantly increased

by sulfur-containing fertilizers. This result is of importance because it indicates a varying concentration of these amino acids in pea proteins. Either the proteins have no rigidly fixed amino acid composition or else the increased sulfur causes an increase in the proportion of the fractions containing more of these acids. The results offer no clue as to how they should be interpreted.

The results of Evans *et al.* with peas are substantiated by work with wheat proteins in the reviewer's laboratory. Rigby & McCalla (70), Gerbrandt (71), and Kasting (72) have demonstrated that gluten may contain widely varying amounts of sulfur, and that this is reflected in the cystine and methionine values. Furthermore, increased sulfur is often accompanied by decreased protein content. Cystine, expressed as a percentage of gluten protein, was increased by at least 60 per cent when sulfur fertilizers were supplied the wheat crop grown on soil very deficient in sulfur, although protein content was either reduced or did not change (70). The variation in the proportion of cystine in the total gluten protein agrees with the results reported by Evans *et al.*

Gerbrandt (71), on the other hand, found a high correlation between protein content and cystine and methionine of wheat from an entirely different source. Thus these results failed to confirm those obtained by Rigby & McCalla (70). Kasting (72), however, did confirm these earlier results when studying samples from the same field plots for later years. Thus, where sulfur is definitely limiting in supply, the cystine content of the wheat and of the gluten appears to be determined by the sulfur uptake and not by the amount of nitrogen absorbed. Kasting obtained the following correlation coefficients in studies for 1946 and 1947, respectively in each case: cystine and sulfur, 0.916¹ and 0.959;¹ cystine and nitrogen, -0.291 and 0.248; methionine and sulfur, 0.78¹ and 0.76¹; and methionine and nitrogen, 0.020 and 0.210. The consistency and levels of these statistics do not permit of an alternative to the conclusion that sulfur uptake by the wheat plant is the deciding factor in determining the cystine and methionine content of the wheat seed proteins. It is now necessary to determine the distribution of these amino acids in fractions of the wheat protein. Kasting has already shown that a fairly high proportion of the cystine occurs in the nongluten nitrogen fraction of flour.

¹ Statistically significant beyond the 1 per cent point.

Most other investigations on cystine and methionine have been part of more general studies. In order to conserve space in discussing the results of such investigations it seems better to deal with all the amino acids rather than with just these two. It should be emphasized that details of the results obtained by many investigators as given in the extensive summary of Block & Bolling (73) are excluded here, although comparisons will be made where these seem necessary.

Barton-Wright & Moran (74) discussed the distribution of 11 amino acids in various fractions of the wheat kernel. Many of the results obtained do not agree well with those given by Block & Bolling (73), this being particularly true for the results with isoleucine. Barton-Wright & Moran report 6.97 per cent for the isoleucine content of whole wheat expressed on the basis of 16 per cent nitrogen in the protein [a practice to which Vickery & Clarke (63) take very strong exception], while Block & Bolling report 3.3 per cent. The values for leucine are decidedly inconsistent, and appreciable disagreement exists with many other results. It should be stressed, however, that Barton-Wright and Moran carried out their studies on milling fractions of wheat and their results cannot be interpreted in terms of wheat proteins.

Doty *et al.* (75) determined the distribution of various protein fractions and amino acids in corn from a large number of single crosses. Significant variations were found in the distribution of protein fractions and in the percentage of amino acids of some of these crosses. It was concluded that the amino acid content of the protein was related to the genetic constitution of the hybrids. It should be emphasized again at this point that with such a complex protein system as that in corn, such differences in distribution of individual amino acids may reflect only the difference in the proportion of the various protein fractions. The data presented do not in any way show that the amino acid content of any specific protein fraction was significantly variable.

Reference has already been made to Chibnall's review (61). His summary table gives results of analyses for 17 amino acids in gliadin and zein. The very high values for glutamic acid and amide-nitrogen in gliadin, and the more or less parallel results for these two determinations in both proteins, are most striking. The amide-nitrogen values agree well with those reported in many earlier

studies. Higher values for the most soluble fraction of gluten have been reported by McCalla & Rose (5) and McCalla & Gralén (8). Despite the fact that gliadin and zein are both prolamines prepared in much the same manner there are wide differences in the amino acid constitution. Space forbids extensive discussion of the detailed results.

There are many other experiments with cereal seeds, but few of them give evidence that is of primary value either to the protein chemist or to the plant physiologist. It is of importance from the nutritional point of view to demonstrate that legumes grown under varying conditions vary in content of the essential amino acids as has been shown by Sheldon, Blue & Albrecht (76). In this report, however, not even the total nitrogen is given, so it is impossible to determine what the variability in proportion of each amino acid may have been. Results, such as these, give even less fundamental information as to the basic distribution of amino acids than do results of studies such as that of Doty *et al.*

On the other hand, McElroy and associates (77, 78) have demonstrated significant variability in the distribution of the essential amino acids as a percentage of the protein in a number of samples of wheat, oats, and barley. Thus either the proportion or composition of the protein fractions differ significantly. Further discussion of data of this type seems unwarranted in this review.

Smith *et al.* (79, 80, 81) have studied the amino acid distribution in the crystalline globulins of hemp, cucurbits, and tobacco seeds. In the first paper (79) leucine, valine, phenylalanine, and tryptophane values are reported. They point out that the tryptophane content of edestin has always played a prominent part in estimations of the minimum molecular weight of this protein. Smith *et al.* stated that the edestin molecule contains three, and not four, tryptophane residues. Edestin is also reported to contain 16 phenylalanine residues and 28 each of leucine and valine. Detailed physical measurements of the other seed globulins were not available. It was concluded that the proteins from the different genera of cucurbits are easily recognized as being of different composition.

More recently, Smith & Greene (80) continued this study and report analytical values for the basic amino acids, isoleucine, threonine, methionine, and cystine. They give a summary table covering these and earlier determinations, and listing the percentage of each amino acid and the number of residues for the minimum molecular

weight of each. In a later note (81) corrected values for isoleucine are given. Smith & Greene (80), however, found that cystine and methionine did not account for all of the sulfur in the crystalline proteins. They believe that the errors involved are not high enough to account for the discrepancies, but point out that Hess & Sullivan (82) feel it is unnecessary to assume that another type of sulfur exists in these proteins. Further studies are reported to be in progress.

Halwer & Nutting (83) showed that cystine disappeared from acid hydrolysates, while Olcott & Fraenkel-Conrat (84) found that cysteine reacts with pyruvic acid in acid solution. Since cysteine was shown to be formed through the reaction of cystine with tryptophane, it seems possible that the failure of Smith & Greene (80) to recover the protein sulfur as cystine and methionine may be explained.

In the review by Chibnall (61) reference was made to unpublished data obtained by some of Chibnall's associates. Macpherson (85) and Rees (86) have since published some of these data. Results for edestin, as well as for zein and gliadin, are given. Macpherson determined arginine, histidine, and lysine and compared his results with those in the literature. He believes that with the newer methods these basic amino acids can be determined with a reasonable degree of certainty as to their accuracy, apart from possible losses in hydrolysis. The high value for arginine in edestin contrasts strikingly with the low values in the two cereal proteins, while the absence of lysine from zein as reported in earlier work is confirmed by the newer methods. The arginine nitrogen of gliadin expressed as a percentage of the protein nitrogen is very close to the values reported by McCalla & Rose (5) for the most soluble fraction of gluten. Rees (86) determined threonine and serine in gliadin and edestin. He also determined the true amide-nitrogen and the ammonia produced on hydrolysis of the proteins in acid. Part of the discrepancies found (0.70 per cent for gliadin and 0.64 per cent for edestin) was accounted for by ammonia due to decomposition of serine and threonine.

While the foregoing discussion indicates that progress is being made in the study of the chemical constitution of seed proteins, many difficulties have yet to be overcome before entirely acceptable results can be obtained. Undoubtedly the major problem is the hydrolysis of the protein.

PROTEINS OF VEGETATIVE PARTS OF PLANTS

The recent paper of Dunn *et al.* (87) offers a natural transition from seed proteins to proteins of the vegetative parts of the plant. This work involved seeds, etiolated sprouts, and green plants of lupin and soybean. The most striking result of the study is the very large increase in aspartic acid in etiolated sprouts of *Lupinus angustifolius*. The increase was from 5.2 to 25.5 mg. per seed or sprout in 15 days. All other amino acids except histidine decreased during the same time. The decrease was much the greatest with glutamic acid. The trend in soybeans was similar but less marked. The changes in proportions of amino acids in plants grown in garden soil under natural conditions were much less than those that occurred in the dark.

The review by Vickery (3) frequently referred to in an earlier section of this paper also dealt extensively with leaf proteins. The importance of the association between chlorophyll and protein was stressed; a relationship more recently discussed by Sideris (88) in a study of the pineapple plant.

The problems involved in accurate study of leaf proteins are similar to, but in some ways more serious than, those confronting the investigator working with seed proteins. Pigments cause more difficulty, and the preparation of anything resembling "pure" protein is a rarity. Despite the fact that some workers believe the extracted protein of leaves represents the "whole" protein, one may reasonably doubt that studies on such whole protein are representative of physiologically distinct proteins in the leaf. Thus, Lugg & Weller (89, 90, 91) considered protein extracted from the leaves of various species of plants to be representative of the total protein of these leaves. The analytical results for "whole," "extracted," and "residual" protein (89) are very much alike, but one cannot interpret these results in terms of either protein or physiological chemistry. Lugg & Weller believe that losses in amino acids occurring during hydrolysis are small. Lugg (92) had earlier investigated the problems associated with the acid hydrolysis of impure leaf protein preparations. He found that liberation of amino groups as a result of hydrolysis of protein at 99°C. was not at a maximum until after 75 hr., and even then the liberation was not quite complete. Furthermore, by that time, some of the amino nitrogen had been degraded to ammonia. The formation of humin nitrogen was

also recorded. It is difficult to believe, therefore, that hydrolysis of impure leaf proteins can be carried out without appreciable error resulting in later determinations of some amino acids. Values for a number of amino acids in leaves of several plant species are recorded by Lugg & Weller (90, 91).

Crook (93) has also pointed out the difficulties encountered in extracting proteins from green leaves and other vegetative parts. He developed an extensive system of grinding in a triple roller mill and secured 95 per cent extraction. While all methods of extraction cause some alteration in the proteins, Crook believes that this method causes the least.

Wildman & Bonner (94) have made a detailed study of proteins from the leaves of spinach. The leaves were ground in a blender and colloid mill and the slurry centrifuged. The juice was dried in the frozen state, and the dried material designated "whole protoplasm." This material contained nitrogen equivalent to 63 to 72 per cent of the total nitrogen in the leaves. The authors believe that this proportion corresponds to the degree of rupturing of the leaf cells. Thus, the dried material is presumably considered as representative of all the protein in the cells. Cytoplasmic proteins were separated from the whole protoplasm and studied electrophoretically. Two principal fractions were obtained. The first constituted 70 to 80 per cent of the total cytoplasmic proteins and appeared to be electrophoretically homogeneous. The second fraction was electrophoretically heterogeneous and appeared to include a number of individual proteins. The first fraction yielded auxin on treatment with alkali or proteolytic enzymes, and also displayed phosphatase activity. The bound auxin and phosphatase activity appeared to be integral properties of this fraction. The second fraction contained small or negligible amounts of auxin, but the activity of all enzymes except phosphatase was associated with this fraction. While the physiological interpretation of results such as these is undoubtedly affected by the preparation, the application of modern physical methods marks a distinct advance in the study of such proteins. Vickery (3) discusses the dynamic system that must exist with these proteins, and one may conclude that the results presented by Wildman & Bonner support this suggestion of reactivity, even though Vickery expressed doubt that prepared proteins would resemble the original material in the cell at all closely in physical and chemical properties.

The proteins of potato tubers have been extensively investigated in recent years. These differ widely from leaf proteins but may still be considered vegetative, as distinct from seed, proteins. Groot (95, 96) determined the amino acid content of the protein coagulated from potato juice but stressed that such protein does not represent the whole protein of the tuber. Groot and associates (97) have shown electrophoretically that the globulin "tuberin" contains two proteins, and they propose the name tuberinin for the new one. This fraction has the stronger negative charge and is heat coagulable. Slack (98) found that a sample of tuberin had a lysine content of 7.7 per cent, more than double that reported by Groot (95). He considers this value to be more in keeping with the high nutritive value of tuberin. He presented amino acid results for tuberin and for whole potatoes and concluded that the higher nutritive value of whole potatoes over tuberin could not be accounted for by the protein that was not extracted from the tuber in the preparation of tuberin. Since Groot *et al.* (97) showed, however, that approximately 30 per cent of the protein in potato juice was distinct from tuberin, it seems possible that Slack's suggestion that the potato contains substances that are neither proteins nor amino acids but replace or supplement these in the diet may be unnecessary.

Jirgensons (99) investigated potato proteins and found that the character of the globulin could be changed to that of an albumin under experimental conditions. He also found that some of the globulin became less soluble as a result of the same treatment. There were definite differences in the viscosity of the globulin, the albumin, and the more difficultly soluble proteins. Despite the fact that two of these proteins are artifacts under Jirgensons' conditions the study indicates that the natural globulin is heterogeneous.

In reviewing work on a wide variety of plant proteins one is struck by the tremendous advantages of studying these substances by the modern physical methods. Papers presenting results of such studies stand out clearly in this review as marking the greatest advances in our understanding of fundamental physical properties of the plant proteins. The preparation of many of these proteins remains a serious problem, but results such as those reported on a wide range of proteins [e.g. (8, 23, 38, 48, 94)] give the most precise information yet obtained. The greater application of all such

methods offers great hope for a rapid increase in our knowledge of the physical properties of plant proteins.

Extraction and hydrolysis of proteins remain as the greatest difficulties in studying the chemical composition of these proteins. Nevertheless, the newer methods offer tremendous advantages, and the value of accurate chemical data to the protein chemist can hardly be overstressed. Only when much more information on both physical and chemical properties is available will anything approaching a satisfactory understanding of most of these substances be possible.

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ORGANIC ACIDS OF PLANTS¹

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The dramatic advances in knowledge of the metabolism, in animal tissues and bacteria, of the acids which interact in the tricarboxylic cycle have been reviewed extensively (1, 2, 3), and the same may be said in regard to the closely related topic of carbon dioxide fixation in such tissues (3, 4). It is well, therefore, to confine attention to studies of higher plants and fungi. These have so far yielded an imperfect picture of the mechanisms and of the significance of the changes undergone by these acids in plant tissues and this is perhaps due to low activity or stability of the plant enzyme systems or to the presence of inhibiting substances. Although strikingly large and rapid changes in quantity of malic, citric, and isocitric acids have been observed in plant tissues *in vivo*, the enzymes involved in the tricarboxylic acid cycle have been, in almost every case, first discovered in animal, yeast, or bacterial tissue and attempts have then been made, not always successfully, to demonstrate their presence in higher plants.

It is probably right to assume, unless one has clear contrary evidence, that the metabolic interactions and the enzyme systems involved are essentially similar in both plant and animal tissues. Our knowledge and opinions regarding chemical mechanism are thus largely derived from work on animal tissue. Understanding of the physiological significance of acid metabolism is almost equally scanty with respect to both animal and plant tissues.

ANALYTICAL TECHNIQUE

Qualitatively, the major advance of the last decade has been the recognition of the so-called crassulacean malic acid as isocitric acid (5, 6, 7), originally incorrectly regarded as an internal anhydride, malyl-malic acid (15). Occurrence of isocitric acid in fruits of *Rubus* had been demonstrated many years before by Nelson (8 to 11), and although no wide survey has yet been published it seems probable that this acid is widespread in plants. Incidentally there is still confusion and uncertainty regarding the configuration of nat-

¹ This review covers the period from July, 1940 to July, 1948.

ural isocitric acid. Krebs & Eggleston (17) refer to it as L (-) isocitric acid but ascription of the L-configuration to the α -carbon is an assumption based only on analogy with malic and tartaric acids which have this configuration. There is disagreement also regarding the sign of rotation of the free acid given as negative by some (17) and as positive by Pucher and his co-workers (13), who consequently refer to the acid as *d*-isocitric.

Four isomers are produced when the acid is synthesised since there are two asymmetric centres. The Wislicenus-Nassauer synthesis produces nearly equal quantities of each, but in the Fittg-Miller process the D- and L-forms of natural isocitric acid predominate (12).

Krebs & Eggleston (17) have developed a method for determination of isocitric and aconitic acids based on the enzymatic conversion of these acids to an equilibrium mixture with citric acid by aconitase. There is some disagreement regarding the composition of the equilibrium mixture (14, 16, 17, 18).

Enzymatic methods are also used in the technique developed by Krebs, Smyth & Evans for determination of fumaric acid (19). Pucher, Wakeman & Vickery (20) have reviewed their analytical methods and recorded certain improvements in detail. Variants of their citric acid method have been published (21,22) having much the same order of accuracy and presumably special suitability for various animal tissues. It has not been possible to reduce the errors of around 8 per cent in malate determinations by their colorimetric method.

Development of partition chromatography is providing somewhat simpler techniques. Isherwood (23) has developed a silica gel sufficiently nonadsorptive to permit quantitative separation of a wide range of acids including oxalic, lactic, succinic, fumaric, malic, and citric. These can be identified by their R_F values. Paper partition chromatograms have also been used for qualitative separations (24).

CRASSULACEAN TYPE OF METABOLISM

Members of the Crassulaceae (25, 26), Cactaceae (25), Liliaceae (25), Bromeliaceae (27), and Compositae (28, 29), amongst many other natural orders, exhibit the peculiar metabolic behaviour of accumulating an organic acid in their tissues when in the dark. The acid disappears again on illumination and a carbohydrate is

formed, so the normal alternating periods of light and darkness cause periodic fluctuation of acid and carbohydrate content. The amplitude of these concentration changes may be from 5 to 15 millimoles per 100 gm. fresh weight of tissue in *Bryophyllum* or *Sedum* (25, 30). Attention is called to the lack of use of metabolic quotients by plant physiologists in recording their data; much inert wall material is included in the dry weight so that it is no more satisfactory as a basis of reference than fresh weight. Rates of malate formation in Crassulaceae may be as high as 50 to 100 mg. per hr. per 100 gm. fresh weight; translated as Q_{Malate} , using the notation applied to glycolysis in muscle, its value is of the order of 3 to 7.

Recent work has provided much fuller information regarding these diurnal correlated changes in carbohydrate and acid content (30, 31, 32). In the upper (younger) leaves of *Bryophyllum* (30) most of the change in acid consists of loss and gain of malic acid, and change in carbohydrate occurred largely in the starch fraction. Krebs & Eggleston (17) also found change in malate with little change in citrate and isocitrate content and Wood (87) has obtained similar results using *Sedum*. Loss of malate is accompanied by small but significant loss of citrate during daylight and equally small but significant gain of isocitrate.

There is a parallelism, the details of which are not yet quite certain, between the loss of starch and gain of acid during the night; in one experiment loss of 6.7 gm. starch was associated with gain of about 10 gm. malic acid per kg. tissue; this is nearly two moles acid per glucose unit of starch. In leaves of *Sedum* the amount of acid formed per glucose unit of starch has been in general smaller but is commonly over one (cf. also Table III, p. 644).

The explanation of this starch-malate equivalence can be surmised on the basis of carbon dioxide fixation in bacterial tissue and it has now been demonstrated that fixation occurs in the Crassulaceae in two important studies. Thomas & Beevers [paper read before the Society for Experimental Biology in London in January, 1947; quoted in ref. (33)] showed that the normal deacidification in daylight does not take place in *Bryophyllum* when the leaves are surrounded by atmospheres containing 5 to 10 per cent carbon dioxide, and the leaves may gain acid instead of losing it even when illuminated. Bonner & Bonner (18) quite independently confirmed and extended the observation of Thomas &

Beevers that acid formation in darkness is greater in atmospheres enriched with carbon dioxide; some of their data are given in Table I and one should note the very marked effect of the low concentrations found in air.

TABLE I

Plant	Change in acid after 24 hr. at 11°C. m.eq. per 100 gm. dry weight		
	Carbon dioxide concentration per cent		
	0	0.03	10
<i>Bryophyllum fedtschenkoi</i>	+ 8	+83	+89
<i>B. crenatum</i>	+17	+47	+129
<i>Mesembryanthemum edule</i>	+13	+21	+33
<i>Sedum</i> sp.	+25	+23	+53
<i>B. crenatum</i>			
malate only	+ 5	+23	+56
citrate only	+18	+24	+29
isocitrate only	-14	+14	+37

The very low carbon dioxide output from leaves of the Crassulaceae immediately after darkening has been known for a long time (25, 35), and Wolf has shown (34) that small cubes of *Bryophyllum* tissue evolved smaller quantities of carbon dioxide when supplied with pyruvate than when supplied with phosphate buffer alone. Bonner & Bonner suggest reasonably that the simple explanation is that pyruvate combines with carbon dioxide to give oxaloacetate which is reduced to malate.

That carbon dioxide fixation does take place has been made quite certain by later work by Thomas, Ranson & Beevers communicated to the Society for Experimental Biology in March, 1948 (33) from which these authors kindly permit quotation. The most striking feature of this work is the parallel determination of change in acid content and of change in composition of the gas phase in which the leaves were enclosed. Leaves were enclosed in about 10 times their volume of air enriched with 5 to 10 per cent of carbon dioxide and samples of gas were removed at intervals for analysis (cf. Table II).

TABLE II

COMPOSITION OF GAS PHASE SURROUNDING LEAVES OF *Bryophyllum*
EXPOSED IN DARKNESS TO VARIOUS ATMOSPHERES. TIME FROM
START IN HOURS. GAS CONTENTS AS PERCENTAGES

Temperature	Hours from start	Carbon dioxide	Oxygen
22°C.	0	5.22	19.53
	2	4.83	19.44
	5	4.59	19.09
	8	4.14	18.87
	30	5.72	17.00
10°C.	0	6.6	20.3
	3	5.6	19.5
	5	2.3	18.7
	7	0.7	18.1
	24	4.7	12.1

TABLE II—(continued)

QUANTITY OF ACID AS MALATE AFTER VARIOUS TREATMENTS

Treatment	Mg carbon dioxide fixed	Mg malate found	Mg malate calc.
5 per cent carbon dioxide	12°C.	164	469
	28°C.	64	147
	38°C.	45	74
Air	22°C.	120	295
	28°C.	68	107

Changes in gas content of the surrounding atmosphere in 2 typical experiments of Thomas and his co-workers (Table II) show clearly that large quantities of carbon dioxide and smaller volumes of oxygen are absorbed by *Bryophyllum* leaves in darkness. Assuming that for each molecule of oxygen absorbed, one of carbon dioxide is formed from carbohydrate and that this carbon dioxide is fixed together with that absorbed from the external atmosphere, one can get a figure for the total carbon dioxide fixed. In the lower part of Table II are given some of their data of carbon dioxide

fixed, malic acid formed, and malic acid expected on the basis of the hypothesis that each molecule originates from one molecule of a 3-carbon compound plus one molecule of carbon dioxide. There is reasonably good agreement and Thomas justifiably writes the over-all reaction as $C_6H_{12}O_6 + 2 CO_2 = 2 C_4H_6O_6$.

This carbon dioxide fixation clearly explains the extremely low normal rates of emission of the gas during the phase when malate is rapidly accumulating at the expense of carbohydrate. With final loss of carbohydrate reserves, rapid output of carbon dioxide occurs. This respiratory behaviour has been observed in Crassulaceae (25, 35), Compositae (29), and in all cases in succulent plants. It suggests at first that the succulent habit (by inducing exceptionally large internal concentrations of carbon dioxide) forces an equilibrium in the direction of carboxylation and that the lowering of carbon dioxide tension during photosynthesis shifts the equilibrium towards decarboxylation. A process of glycolysis in which pyruvic acid is diverted after carbon dioxide fixation to malic acid has energetically much in common with the process in which lactic acid is formed in animal glycolysis. "Malic

TABLE III
ACID CONTENT OF LEAVES OF *Bryophyllum* CULTURED IN DARKNESS
FOR VARIOUS TIMES (32); ALL QUANTITIES GIVEN
AS GM. PER KG. FRESH WEIGHT

Temperature	Hours in darkness	Malate	Citrate	Isocitrate	Starch	Soluble carbohydrate
	Initial state	1.5	1.3	9.3	12.1	5.1
20°C.	20	10.0	5.4	7.9	3.3	5.0
	65	3.9	6.5	9.2	3.8	4.9
	140	2.8	6.2	9.2	3.9	5.1
9°C.	20	17.0	3.4	8.7	0.7	4.8
	65	11.8	7.1	9.3	0.3	5.7
	140	8.0	10.4	10.5	0.1	5.3
1°C.	20	8.1	3.1	13.0	5.9	5.1
	65	9.5	5.1	13.6	3.1	6.4
	140	9.8	6.7	9.8	0.7	5.9

acid glycolysis," however, is inhibited under anaerobic conditions in Crassulaceae and beet.

Pucher and co-workers have investigated changes in acid and carbohydrate contents of leaves kept in continued darkness at different temperatures (32); certain features of their data are summarised in Table III. Attention may be called to certain points: protein and soluble nitrogen fractions scarcely changed in quantity; there was little change in soluble carbohydrate; carbohydrate losses were too small to account for acid gains during the first 20 hr. unless one assumes formation of two malate molecules per hexose unit which presupposes carbon dioxide fixation; at 9°C. between the twentieth and one hundred fortieth hour there is a loss of malate of 9 gm. per kg. and gain of 7 gm. citrate plus 1.8 gm. isocitrate per kg. leaf tissue associated with negligible changes in carbohydrate and protein fractions. The over-all reaction between malate and citrate must thus read 3 malate \rightarrow 2 citrate and not 2 malate \rightarrow 1 citrate + 2 CO₂ which represents the simplest scheme derived from the tricarboxylic acid cycle. The very wide divergence between concentrations of citric and isocitric acids found and the equilibrium concentration ratio of roughly 20:1 is noteworthy but both this and the apparent interconversion of malate to citrate and fluctuations in isocitrate are at present unexplained in detail.

PLANTS WITHOUT CRASSULACEAN METABOLISM

Little new information is available. Rhubarb leaves (7, 36) cultured in water, after 25 hr. continuous illumination, show an increase in acid content, almost all malic, which amounts to roughly 13 per cent of the amount initially present; a somewhat similar increase is found when they are grown in glucose solution. Earlier workers had also noted an increase in acidity on illumination in contrast with the behaviour of the Crassulaceae (38).

Leaves of rhubarb and *Nicotiana*, like many fruits, show a steady loss of acid in long continued darkness (37, 39). In *Nicotiana* loss in total acid is associated with an apparent conversion of malate to citrate; as in *Bryophyllum* (see Table III), more than one molecule of citrate is formed for every two molecules of malate lost.

Fagopyrum, which is an oxalate plant showing small diurnal periodicity in pH, has been reinvestigated (40), but its behaviour

remains somewhat obscure as gains in total acid are recorded during both day and night which evidently is not consistent with long continued diurnal periodicity; either the reported periodicity or the continued gain in acid content would appear to be due to sampling errors.

The tendency to assume that the striking citric acid formation by certain fungi, species of *Aspergillus* and *Penicillium*, is brought about by reactions of the citric cycle may be understandable, but should be much further investigated. Active citric acid producing strains have been shown to have an almost negligible zymase activity (41). This, coupled with the well-known occurrence of glucose-oxidase in these fungi, does suggest that the early stages of glycolysis may be oxidative. Feeding experiments have so far proved rather unhelpful; certain inhibition reactions are as yet unexplained. Allsopp (42), for example, found that oxalic acid formation in a strain of *Aspergillus niger* was inhibited by lactic acid. Roberts (88) writes that a strain of *A. niger* which produced citric acid rapidly from sugar failed to form any when fed with *cis*-aconitic acid, which should be its immediate precursor on generally accepted hypotheses.

On the other hand Lynen (43) feeding yeast with deuterium-labelled acetate, CD_3COOH , found both succinate and citrate containing deuterium. Carbon dioxide fixation using isotopic carbon has been shown in several fungi (3), but large scale fixation, comparable with that of the Crassulaceae and propionic acid bacteria, has not been observed in mould fungi, though their growth rates are markedly reduced in atmospheres kept as free as possible from carbon dioxide.

ENZYME SYSTEMS ISOLATED FROM HIGHER PLANTS

Clear identification of all the enzyme systems involved in the tricarboxylic acid cycle has not been obtained in any one plant, though certain components have been detected in different plants. It is characteristic of vacuolate tissues of plants that extracts, homogenates, acetone preparations, or brei show negligible total respiration and most components of the acid cycle cease reacting. Henderson & Stauffer (44), however, have obtained active material from minced tomato roots.

Presence of pyruvate and of pyruvate and oxaloacetate decarboxylases can be regarded as reasonably clearly established (45,

46) in a number of plants, such as barley, pea and bean seedlings, tomato roots.

Citrogenase, which, in certain animal tissues, catalyses the interaction of pyruvate with oxaloacetate or acetoacetate, with resultant production of citrate, has not been detected in plant tissues (21, 47). Rapid conversion of malate to citrate occurs in certain leaves (37, 39), but the enzyme systems involved are not known although the reaction apparently is of the type 3 malate \rightarrow 2 citrate without carbon loss in the form of evolved carbon dioxide.

Occurrence of aconitase and fumarase is well established in many leaves and seeds (48 to 52). Berger & Avery report that a fresh extract of *Avena* coleoptiles can dehydrogenate (in presence of methylene blue and TPN) isocitrate, *cis*-aconitate, and citrate; so that evidently both isocitrate dehydrogenase and aconitase are present: ammonium sulphate precipitation causes a two-thirds loss of reactivity of citrate but little change in that of *cis*-aconitate, indicating a loss of β -aconitase activity (52). Jacobsohn (51) also considers that there are two aconitases but this view is not very generally held.

Malic, lactic, isocitric, alcohol, and glutamic dehydrogenases have been detected in a variety of plant tissues (53 to 57); most frequently this recognition has been a relatively uncritical dye decoloration in Thunberg tubes, though ketoglutarate formation from glutamate has been observed in enzyme brei from oat embryos (57), and ketoglutarate formation from citrate was observed in *Cucumis* seed (53). This action was later recognised (14) as due to mediation of aconitase and isocitrate dehydrogenase. The immediate product of action of the latter, oxalosuccinic acid, has not been isolated from plant tissues, nor is the presence of oxalosuccinic decarboxylase (58) known.

Succinic dehydrogenase activity in extracts or brei from oat coleoptiles, spinach leaves, and certain other tissues has not been detected; it hardly seems likely that this enzyme is completely absent, particularly since succinic acid in small traces is present in a very wide range of plant tissues (59). That this enzyme is present is claimed on the basis of malonate inhibition data.

Bonner (55) found an accumulation of succinate in coleoptiles treated with 0.001 *M* malonate, and with supply of fumarate in addition to malonate a still larger succinate accumulation was found under aerobic conditions, which is considered evidence for

completion of the Krebs cycle. Laties (60) also found succinate accumulation in malonate-poisoned barley roots, and labelled carbon dioxide was recovered in the carboxyl of succinate.

Malonate inhibition phenomena of living plant tissue are complex; many workers find marked inhibition of respiration and associated processes of growth or salt uptake (54, 55, 60), but many find no inhibition (62, 63, 65, 66). Bonner & Wildman (54) show that inhibition is virtually absent when the pH is 6 or more and at its maximum at or below pH 4, which they correlate with dissociation of the malonic acid, which is supposed to penetrate only as undissociated molecules. Turner & Hanly (61), however, find that carrot slices when freshly cut are inhibited at pH 6 but on aging and washing the same malonate markedly stimulates respiration; they find moreover that malonate inhibition is not reversed by supply of fumarate or succinate. Machlis (65) also finds no reversal of malonate inhibition by malate, succinate, and fumarate, while Bonner & Wildman, and also Laties, find reversal. Maleate, a similar inhibitor to malonate, at pH 3 to 4 is reported by Lundegårdh to have a stimulating rather than inhibitory effect on respiration and he does not consider that there is evidence for organic acid catalysis in wheat roots (62).

Influence of pH on acid utilisation by living cells is probably very complex. Smythe has shown that yeast consumes pyruvate aerobically more rapidly at pH 7 than at pH 5.6 and that little anaerobic utilisation of pyruvate occurs at these pH values. At pH 2 to 4, however, there is rapid anaerobic utilisation (67). An explanation of these results based on permeability relations must postulate better penetration to aerobic enzyme centres at high pH coupled with failure to reach anaerobic centres and a rapid penetration to all centres at low pH. No complete explanation of these malonate and pyruvate effects is possible at present; it would be unwise to assume that malonate effects are solely due to succinoxidase inhibition.

The complete coenzyme I and II linked dehydrogenase systems have been known for some years to require presence of diaphorases. Occurrence of diaphorase in seedlings has been reported (64).

A recent very careful quantitative study (68) has been made of the occurrence of coenzymes I and II and flavine-adenine-dinucleotide (FAD) in various plant tissues including the leaves of *Stellaria*, *Trifolium*, and potato. Quantities present were of the

order: FAD 0.2 to 0.4 $\mu\text{g.}$, coenzyme II 1 to 8 $\mu\text{g.}$, coenzyme I traces, all expressed per gm. fresh weight. These concentrations are considerably less than those found in yeast and muscle.

PHYSIOLOGICAL SIGNIFICANCE OF ORGANIC ACIDS

It may be that the main role of acid metabolism in plants is to facilitate the building of high energy phosphate bonds. Catalytic effects of component acids of the tricarboxylic cycle are not observable in plants possibly because the large amounts of acids normally present mask effects due to a small extra supply.

Extra supply of the acids of the cycle undoubtedly causes increased gas exchanges in a wide range of plant tissues such as roots of barley (65), wheat (62) and tomato (44), beet (66), potato (69), oat coleoptiles (70 to 73), and various leaves and fruits. This is not an example of catalysis in beet (66) where the acid is consumed, and the same may be true of the other tissues examined.

James and his colleagues (74, 75) have shown that in barley the lactate \rightleftharpoons pyruvate system is coupled with ascorbate \rightleftharpoons dehydroascorbate system, and he suggests that this may be a pathway of hydrogen transfer. Ball's argument (76) that the Szent-Györgyi reaction is a "blind alley" applies equally here. James' scheme could only be a facilitation mechanism if the $\text{DPN} \cdot \text{H}_2$ which reduces pyruvate to lactate is unable to reduce dehydroascorbate to ascorbate; but presumably DPN is also the intermediary in lactate reduction of ascorbate though it is conceivable that some other enzyme system may operate.

Plant acid metabolism is undoubtedly closely associated with uptake of mineral ions from soil. Ulrich's important study (77) has shown that uptake of excess of inorganic cations over anions is associated with formation of an approximately equivalent quantity of organic acid, and conversely uptake of an excess of anions results in disappearance of an equivalent of organic acid. Excess cation uptake occurs from potassium bromide and, associated with this, is an increase in respiration and a decrease of the R.Q. below unity since organic acid is being produced. In contrast barley roots supplied with calcium bromide absorb only traces of calcium and considerable amounts of bromide and the excess anion absorption is balanced by organic acid loss which thus causes a rise of R.Q. above unity.

Machlis (65) has studied bromide uptake of barley roots in the

presence of respiratory inhibitors with and without further additions of malate, succinate, fumarate, and citrate. Iodoacetate inhibition of respiration also inhibits bromide uptake and these inhibitory effects are removed by supply of 0.05 *M* malate, citrate, succinate, etc. Inorganic salts, such as sulphate of the same potassium content as the succinate and citrate had a similar effect to the organic salts in enhancing respiration and salt uptake in the absence of inhibitor, but sulphate did not cause reversal of iodoacetate inhibition as did citrate and succinate. Evidently respiratory processes associated with 4 carbon acids can promote salt accumulation when glycolysis is blocked by iodoacetate.

Malonate inhibition was only found in very concentrated solutions and then affected salt uptake and respiration; these inhibitions were not removed by a supply of 4 carbon acids at the same concentration. Hoagland has reviewed work of his school on interrelations of salt uptake and metabolism (78).

Pucher and co-workers (32) have extended the earlier work of Clark (80) and show that in *Bryophyllum* and *Nicotiana* nitrogen supplied as nitrate causes organic acid accumulation in much larger quantity than when supplied as ammonium. In the latter case, there is a relatively small excess inorganic cation accumulation as the ammonium ions are used in protein synthesis. There is a rough parallelism between contents of malate, citrate, and isocitrate in *Bryophyllum* and *Nicotiana* grown in nitrate and ammonium culture, the nitrate plants containing about four times as much acid as those supplied with ammonium in the case of *Bryophyllum*. The occurrence of a large excess of inorganic cations over inorganic anions in *Nicotiana* (ratio about 400:70) had been noted earlier (79) together with the fact that the balance was maintained by a correspondingly large organic anion content.

It seems possible that increasing pH may "stimulate" acid production, but there is no clear evidence of this. One may note, for example, that itaconic acid production by *Aspergillus terreus* is at its maximum at pH around 2 and is inhibited by increase of pH (85). On the other hand, strains of *A. niger* produce oxalate from citrate, succinate, etc., when these are supplied as sodium salts but no oxalic acid is formed from the free acids which suggests some form of cation trapping.

Many investigators have noted correlation of growth phenomena with 4 carbon acid metabolism, but the mechanism by which

these acids exercise these effects remain obscure. Some studies originated with the observation that rates of streaming of oat coleoptile cells are significantly increased by indoacetic acid in the presence of 0.001 *M* malate or fumarate (84). Correlation of extension growth in *Avena* coleoptile with respiration has been frequently observed (70 to 73). Thimann & Bonner (81) have recently shown that iodoacetate inhibition of such extension growth is eliminated by simultaneous supply of pyruvate, malate, and related acids. This seems to indicate that extension is connected with 4 carbon acid respiration rather with earlier phases of glycolysis which are presumably interfered with by iodoacetate.

Some interaction between indoacetic acid (IAA) and the 4 carbon acids is claimed to exist (70 to 73, 84), but failures to find effects of IAA on acid metabolism have been reported also (82). It seems clear, at least, that IAA does not influence the activity *in vitro* of any of the dehydrogenase systems isolated (52).

The influence of this group of acids on growth may reside simply in their essential role as promoters of a supply of respiration energy in some such suitable form as active phosphate. The link between energy made available in respiration in plants and processes of growth or secretion is still to be sought.

The necessity for completion of the tricarboxylic acid cycle in *Neurospora* is demonstrated by discovery (83) of a mutant strain which will grow when supplied with malate, succinate, fumarate, ketoglutarate, or the corresponding amino acids, but which will not grow on citrate, isocitrate, *cis*-aconitate, lactate, tartrate, glycollate, pyruvate, and (surprisingly) oxaloacetate. Lewis (83) suggests a genetic block between isocitrate and ketoglutarate as the explanation of these results with the subsidiary hypothesis that oxaloacetate is ineffective owing to its decomposition on the outside of the fungus. The interconversions may be essential features of amino acid and protein synthesis rather than an essential link in carbohydrate oxidation.

A feature of plant acid metabolism which should be remembered is that the high concentrations recorded in plants (0.05 to 0.2 *N*) probably represent concentrations in the vacuole and one cannot estimate the concentrations surrounding active enzyme systems. The respiration rate of beet slices containing about 0.02 to 0.05 *N* malate plus citrate inside the tissue is almost doubled by bathing the outside of tissue slices with 0.0005 to 0.001 *N* malate,

citrate, or succinate (66, 69). Evidently external acid penetrates more easily to enzyme systems than vacuolar acid. Stimulation of respiration by externally supplied malate, etc., has been observed in other tissues. The suggested explanation (66) is that the vacuolar boundary is less permeable than the external plasmatic membrane. Respiration rates and rates of change of plant acid concentrations in live tissue may thus be largely controlled by permeability or secretion phenomena and an increase of respiration caused by malonate, for example, may be connected with cation uptake, like those noted by Ulrich (77), or may be due to altered permeability of a vacuolar membrane rather than to a specific effect on an enzyme.

CONCLUSION

The theory of carbohydrate oxidation is far less complete in plant tissues (other than yeast) than in animal tissues and the manner of its energetic linkage to essential processes of the living plant is a matter for future research. This review has not attempted to consider the interactions between the tricarboxylic acid cycle acids and the protein metabolism which was clearly recognised and discussed by Chibnall (86). This connection has been emphasised by recent work on transamination (57), which has been shown to occur in plant tissues at rates comparable to those obtaining in animal tissue.

The major advance reported here is the new understanding of the characteristic Crassulacean metabolism which follows from the impressive carbon dioxide fixation demonstrated by Thomas and his colleagues and by Bonner & Bonner in plants of this type. It is increasingly clear that extension growth, salt uptake, and possibly other secretory processes can be maintained by a 4-carbon acid system even when glycolysis is blocked by inhibitors.

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MINERAL NUTRITION OF PLANTS^{1,2}

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In preparing the present review, consideration has been confined to a few general aspects of the problem. In reviewing papers bearing on the selected topics, those which indicate the general trend and progress of research have been selected, and comment upon contributions such as those bearing on the mineral nutrition of lower plants and on inorganic plant poisons is necessarily omitted.

AVAILABILITY OF ADSORBED IONS

Although the influence of the chemical status of the soil on the intake and accumulation of ions is beyond the scope of this review, it is becoming increasingly evident that the presence of charged surfaces in the rhizosphere may have a profound influence on the intake of ions. This consideration is especially pertinent in view of the prevailing evidence that ion exchange phenomena are involved in the adsorption of ions on the root surface and their movement across the cell membrane (45).

The work of Marshall and his colleagues (48) using clay membrane electrodes to measure the activity of cations adsorbed on colloidal clay is giving added impetus to the importance of an adsorption surface in conditioning the cationic environment of a plant root. Calcium adsorbed on a suspension of montmorillonitic clay colloid to the saturation capacity may have an activity of only about 0.0002, whereas sodium adsorbed under comparable conditions may have an activity 20 to 25 times as high. These figures are indicative of the relative ease of release of these two cations to the plant root. In line with the observations of Mattson (49) and Mehlich (50), Marshall points out that the relative energy of retention for the various cations varies widely with mineralogical composition of the colloid. At comparable levels of adsorption,

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² This review covers the period from October, 1947 to October, 1948.

calcium adsorbed on a kaolinitic clay may be 10 to 20 times as readily available to the plant root as compared with calcium held by a montmorillonitic clay. However, the ratio activity-of-sodium:activity-of-calcium is always greater for all clays than the ratio sodium-adsorbed:calcium-adsorbed. The qualitative cationic distribution at the root surface in a mixed system of monovalent and divalent ions will be far different than that on the surface of the clay. Furthermore, this relationship between proportions of cations adsorbed and proportions of cations active will shift markedly with type of colloid involved. That is, intake of cations by the plant is fundamentally conditioned by the presence of a charge surface in the substrate. Availability of potassium is further complicated by its tendency to become fixed in the crystal lattice of the clay colloid [see (8)].

The order of ease of release of cations from a given type of soil colloid is sodium > potassium > magnesium > calcium > hydrogen (14). This serial relationship is related to the relative orientation and oscillation amplitudes of the respective cations in the diffuse double layer about the adsorption surface (62). Thus, increasing proportions of adsorbed sodium or potassium on the clay particle normally decrease the replaceability, or availability to plants, of the adsorbed magnesium and calcium. Correspondingly, by virtue of the complementary ion effect a given level of adsorbed potassium is more available in the presence of a preponderance of adsorbed calcium than in the presence of a preponderance of adsorbed sodium; and, a given level of adsorbed calcium upon clay particles is more active in the presence of appreciable proportions of adsorbed hydrogen than when an equal amount of sodium is adsorbed.

Numerous investigators have shown that calcium is virtually unavailable to plants if about 50 per cent of the cation exchange capacity is satisfied with sodium or potassium (9, 66, 83, 90). Ratner (66) has even concluded from his data that at higher levels of sodium saturation of the soil exchange complex, the soil colloid may actually remove calcium from the root cells causing death from calcium deficiency. Bower & Turk (9) have shown that high levels of adsorbed potassium are just as effective as sodium in preventing the intake of calcium and magnesium by plants. Thorne (83) found that the addition of calcium carbonate to a system containing a relatively high level of adsorbed sodium, actually ac-

centuated the adverse effect of sodium. The complicating effect of added calcium carbonate on the nutrition of the plant appeared to be related to the associated decrease in intake of phosphorus.

Arnon and his colleagues (1, 2) have contributed further evidence of the important effect of an adsorption surface in the substrate on ionic intake and accumulation by their studies in which the nutrient ions were adsorbed on synthetic exchange resins. The relative ease of release of nutrient cations adsorbed on the resin used was of the same order as that for montmorillonitic colloid, but the magnitude of the energy of retention appears to be higher for the divalent cations. In the earlier study (1) intake of calcium and magnesium from this substrate was virtually precluded unless a dilute solution of potassium nitrate was added to the culture medium. The apparent intensity with which this resin (Amberlite 1R-100) withheld calcium and magnesium from the plant as compared to the effect of natural clays is probably related to the fact that the relative energy of retention of hydrogen ion by this resin is intermediate between potassium and magnesium whereas the energy of retention of hydrogen ion on the soil particles is found to be higher than that of calcium. Hence, hydrogen ions arising from the root surface would be more readily exchanged for divalent nutrient cations from the clay than from the resin. The ameliorative effect of added potassium nitrate in the ion exchange resin study was undoubtedly the result of the exchange equilibria between ions adsorbed on the solid phase and those in the liquid phase. The activity of calcium and magnesium ions was sufficiently increased thereby to permit the intake of these ions by the roots. These studies further emphasized the importance of the complementary ion effect in the ease of release of adsorbed cations and the readiness with which physiological calcium deficiency may develop. In line with the above, Vlamis & Jenny (91) reported a case in which plants showed calcium deficiency on a serpentine soil, even though the clay fraction contained 5.5 m.eq. exchangeable calcium per 100 gm.

The foregoing does not specifically emphasize the importance of "contact exchange" between the clay surface and root surface, since it is apparent that the presence of charged surfaces in the rhizosphere exert an influence whether or not the oscillation amplitudes of hydrogen ions on the roots actually intermingle with those of nutrient ions adsorbed on clay particles. Burd (17) has recently

discussed this point. The oriented water molecules surrounding the adsorbed ions constitute an interphase between the soil solution and adsorption surfaces on roots or clay particles. Thus, contact exchange readily takes place when these respective interphase layers commingle, but ionic exchange between hydrogen ion on the roots for nutrients adsorbed on the clay still takes place when these respective interphase layers are separated by liquid phase, i.e., soil solution. Burd (17) emphasizes the importance of carbon dioxide arising from root metabolism as an agent in the transference of hydrogen ion from the interphase at the root surface towards that of clay particles with the exchange for nutrient cations. Mehlich & Reed (52) also stress the importance of carbon dioxide or bicarbonate ion in mobilizing hydrogen ion to effect cationic interchange between root surface and soil colloid. They set forth a hypothesis that specificity in cationic intake among different plants is related to the inherent capacity of the roots of a given species to produce carbon dioxide. Increasing carbon dioxide production yields, in effect, a higher concentration of hydrogen ion, effecting greater replacement of nutrient cations from the colloid. Indirect evidence is cited to support this concept, but its validity must await further experimental verification, especially as to its applicability to alkaline soils.

INTAKE AND ACCUMULATION OF IONS

Although extensive data are available concerning cationic exchange phenomena on soil colloids, information concerning adsorption phenomena at the root surface is indeed meagre. Lundegårdh (44) has presented an acceptable picture of the negative valence forces on roots active in cation adsorption, but no information on the relative energies of adsorption of the various cations by different root surfaces is available to complement that with respect to the soil colloids. The problem is exceedingly complex because of concurrent transference of cations across the root membrane. Relative rate of net accumulation of the various cations inside root cells is the resultant of three processes: (a) relative intensity of adsorption of the nutrient cations on the root surface, (b) relative rate of transference inwardly across the membrane, and (c) relative rate of transference outwardly across the membrane. As pointed out by Lundegårdh (45) and Burström (18) in previous reviews, the first stage is of primary importance. Holm-Jensen

et al. (34) obtained data using radioactive isotopes indicating that potassium and sodium are strongly held in the cell wall. Olsen (61) has also noted that potassium may be adsorptively bound in leaf cells of beech. Unfortunately, there is virtually no information on the relative energies of retention of the various nutrient cations by the root surface, or on the role of the complementary ion effect in modulating the activity of the adsorbed cations.

Studies on the adsorption of ions by dead tissue may not be very illuminating with respect to the phenomena of ionic intake. Potassium can be quantitatively leached from ether-killed barley roots in contrast with the capacity of live roots to maintain an inward gradient (16). Jacobson & Overstreet (35) postulate that for compounds or reactive groups existing in plant cells and capable of fixing or retaining inorganic ions from the culture medium in exchange for equivalent ions released by the plant, the assumed internal constituents must have the following properties: (a) they must be related to the oxidative metabolism of the plant since there is a relationship between ion absorption and oxygen tension; (b) they must form compounds with ions such as those of potassium, rubidium, calcium, bromide, nitrate, sulfate, etc., in which relatively strong bonds retain the ions against an outward gradient; (c) they should account not only for the intake of nutrient anions and cations, but also for a wide range of ions of the same sign; and (d) they must be highly unstable, even though they may bind the ions strongly, since the ions may pass into free solution in the vacuole and the adsorptive powers are largely lost on death.

Jacobson & Overstreet (35) studied intake and loss of radioactive strontium and iodide by barley roots to gain some insight into the nature of ion fixation within the plant. Many of the determinations were carried out at 0° C. in order to avoid complications due to longitudinal translocation. Observations on live roots at 0°C. and 25°C. indicated the marked dependence of intake of radioiodide on metabolic activity. There was marked difference between behavior of iodide and strontium in that the intake of iodide increased by a factor of 7.8 for the 25°C. rise in temperature, whereas intake of strontium increased by a factor of only 1.5. This may imply that metabolic activity is more essential to the intake of iodide than of strontium. The difference between absorption of

iodide and strontium was further emphasized by observations on ether-killed roots. Dead roots show relatively little intake of iodide at 25°C., whereas they were found to take in more strontium at 25°C. during 15 min. than do live roots. No "accumulation" of iodide occurred in dead roots, whereas a large "accumulation" of strontium over the external concentration was found for the killed tissue. This is a further implication of the essentiality of metabolism for intake of iodide in contradistinction to that of strontium.

Dead roots were found to release absorbed ions to the external medium much more rapidly and completely than live roots. The data suggest that strontium is held differently in live than in dead roots; but even in the living tissue, the exchange curves indicate that no appreciable fraction of strontium or iodide is held in a nonexchangeable form. Jacobson & Overstreet (35) conclude that ion-binding compounds are relatively labile and are produced during metabolism in connection with aerobic respiration or as products of metabolic synthesis. It is pertinent to note that their observations on intake of strontium and iodide by excised barley roots are not at variance with Lundegårdh's concept (44) that the energy arising from salt respiration is primarily involved in the intake of anions, whereas cations enter by means of exchange phenomena with internally produced hydrogen ions. This study (35) corroborates earlier work by the same authors using Rb⁸⁶ and P³² (63). It is a commendable contribution towards the elucidation of one of the most fundamental processes in plant nutrition.

An important contribution towards evaluating the role of metabolism in ionic intake has been made by Robertson and colleagues (54, 70). They have given further study to the Lundegårdh theory (44) that salt respiration primarily affects intake of anions. To wit, when the hydrogen atom liberated by the dehydrogenase phase of respiration reaches the cytochrome system, the cytochrome picks up the electrons and the hydrogen ion is freed to exchange for external cations. The resulting ferrocyclochrome, or some analogous electron carrier, effects movement of the electron to the outer cell surface where the electron is lost via cytochrome oxidase to externally supplied oxygen; and the oxidized ferricytochrome, or analogous component, acts as an inward carrier for anions. Robertson & Wilkins (70) point out that if the Lundegårdh theory is valid, the maximum rate of anion accumulation in

the cell should take place when each electron leaving via the cytochrome system is exchanged for an anion from the external medium. Assuming that respiration is proceeding by the cytochrome system, all molecular oxygen involved in the process becomes combined as water, and, therefore, in line with the study by Ball (5) each molecule of oxygen requires four electrons and four hydrogen atoms. Hence, the maximum rate of salt accumulation should be 4 gm. mole of monovalent salt accumulated for each gram mole of oxygen used, or, salt accumulation/salt respiration = 4.

Robertson & Wilkins (70) made a large number of determinations on the rate of intake of chloride in relation to rate of respiration and determined the value of this ratio for carrot tissue. The experiments showed that the rates of both processes are dependent on the concentration of the external solution. However, salt respiration reached virtually its maximum value when the external concentration of potassium chloride was only 0.01 molar; whereas the maximum rate of chloride intake was reached at about 0.04 molar potassium chloride. When the rate of neither process was limited by external concentration, the number of molecules of salt accumulated is of the same order of magnitude as the number of electrons eliminated by salt respiration: e.g., the value of the aforementioned ratio tended to approach a value of four. Robertson & Wilkins consider these observations to be consistent with Lundegårdh's theory; and they have presented a clear and concise exposition of this theory together with a commentary on supporting evidence. Nevertheless, they discuss their results in terms of "salt respiration" rather than as "anion respiration" as set forth by Lundegårdh (44). It should be noted that the hydrogen arising from the dehydrogenase of the respiratory system is converted to hydrogen ion by the action of ferricytochrome. This constitutes an initiation of the cation exchange process involved in the intake of nutrient cations and implies that respiratory activity may condition cationic intake; even though it has not been necessary to hypothesize a complex intermediate transport system as in the case of anion intake. Since hydrogen ions may arise in the plant cell as a result of other processes than that of electron capture by ferricytochrome, it is indeed dubious that a stoichiometrical relation between respiration and cation intake should be found. Available evidence (33, 44, 80) supports this conclusion.

PHYSIOLOGICAL ROLE OF THE NUTRIENT ELEMENTS

It is disconcerting to note that this fundamentally important phase of the mineral nutrition of plants is attracting relatively little active investigation. There have been several valuable contributions to this aspect of mineral nutrition during the past year, and it is to be hoped that the number will increase.

Potassium.—The function of potassium in plants continues to be intriguing, since virtually nothing specific is known concerning the role of this major inorganic constituent in plants. Cooil (22, 23) and Cooil & Slattery (24) have reported their intensive studies on the response of guayule to deficiency and excess of potassium. It was noted (22) that regardless of the level of potassium supply, the equivalent content of cations per unit weight of leaf tissue tended to be a constant. This same phenomena has been noted for several other species of plants (6, 52, 93, 95). Thus, a low level of potassium in the plant is associated with accumulations of other cations; and, hence, the difficulty of distinguishing between responses directly associated with an inadequacy of potassium and those associated with accumulation of other cations. No adverse effects on growth were noted for the supposedly excess level of potassium (12 m.eq. per 1.) even though the mature leaves of these plants contained 10 per cent potassium in the dry matter. However, it was noted that there was a fairly close relationship between potassium content of the plants and growth in diameter of shoots, thus confirming the essentiality of this element for cambial activity.

When the effect of level of potassium supply on nitrogen and carbohydrate constituents of guayule was ascertained (24), the observations were predominately in line with much previous work on other plants. Reducing sugars in the leaves were consistently higher for the low potassium treatments. A parallel observation on *Ocimum canum* was reported by Kalinkevich (37). Cooil & Slattery (24) found that in all tissues of low potassium guayule plants, the reducing sugars were a relatively high proportion of the total carbohydrate, suggesting a relation between activity of potassium in the tissues and degree of condensation of fructose to fructosans. Amide-nitrogen and α -amino nitrogen fractions were found to be highest in expanding leaves containing the least potassium; but there was little consistent trend with potassium levels of the content of insoluble and sap soluble organic nitrogen.

This reviewer concurs with Cooil & Slattery that the probable

role of potassium is that of conditioning the action of specific enzymes. Information on this point is as yet meagre, but Snellman & Erdös (77) have shown that potassium-myosinate behaves quite differently from calcium-myosinate; and it has been shown (39) that potassium has a specific role in activating enzymatically controlled energy transfers involving the phosphate esters. Calcium was found to be antagonistic to the effect of potassium (10). Cooil & Slaterry (24) found that the degree of condensation of reducing sugars to inulin and levulins was significantly related to the ratio: potassium/soluble calcium.

Calcium.—Several studies (28, 64, 96) have further emphasized the importance of calcium for the development of apical meristems. Haynes & Robbins (28) found that calcium and boron are two essential components of the environment of each root. Other nutrients may be deficient from part of the root system but as long as they are supplied to another portion of the root system, translocation appears to be adequate to keep the unsupplied portion healthy. Brady *et al.* (12) have shown that the filling of peanut fruits is quite sensitive to the supply of calcium; a high level being essential for well-filled pods. Due to the complementary ion effect, applications of potassium to the fruiting zone or rooting media were depressing to fruit filling. Mehlich & Reed (51) have shown that because of the sensitivity of the peanut to calcium availability, a much higher level of calcium saturation is required on montmorillonitic soils to produce high quality peanuts than on kaolinitic soils, because of the greater ease of release of the calcium ion from the kaolinitic colloid. The specific metabolic role of calcium in the above instances was not amplified.

Cooil (23) found that the excess of cations over inorganic anions in the leaves of guayule was greatly in excess of the non-volatile organic acids present. If the carbonate content of the leaves was added to that of the organic acids, the above difference was largely accounted for. Cooil found that the carbonate was present in the leaves as calcium carbonate and was confined to the epidermal trichomes. This observation emphasizes how meaningless a total calcium determination may be towards evaluating the activity of calcium in metabolic processes.

Magnesium.—Although there have been few contributions of late towards an understanding of the role of magnesium in the metabolic processes of plants, it is becoming increasingly evident

that magnesium deficiency may be a prime consideration in crop production. Chlorosis and other abnormal pigmentation patterns of leaves associated with magnesium deficiency have been recently reported on pears (27), apples (19), and tomatoes (29, 58). It is frequently noted that magnesium deficiency is accentuated by a high level of potassium supply (11, 19, 27); a low supply of available magnesium in the substrate is usually involved (29, 58).

Sodium.—Sodium has not been established as an essential element for the growth of economic plants, but numerous observations (40, 72, 95) have shown that it may have beneficial or stimulatory effects. Lehr (40), and Sayre & Vittum (72) state that it is not possible to obtain maximum growth of sugar beets without a supply of sodium. Wallace *et al.* (95) confirm much previous work to the effect that sodium may be especially beneficial to plants when the supply of potassium is limiting. There is marked variation among species in their response to sodium, and this may be related to the facility with which sodium enters the plant. Wallace *et al.* (94) studied the sodium content of a large number of species; and, as a consequence, set up a classification of plants on the basis of their sodium content: (a) plants that did not accumulate sodium (b) plants that accumulated sodium if it were present in abundance; (c) plants that accumulated sodium whether it were abundant or not; and (d) plants normally high in sodium. Plants in the third and fourth groups would be the most probable ones to show a beneficial response to sodium. Beets would be classed in the latter groups, and it is pertinent to note that they belong to the Chenopodiaceae, a family that is predominantly halophytic.

Raleigh (65) obtained a marked response of table beets to added sodium chloride in Hoagland's culture solution. Addition of potassium chloride to this nutrient solution was just as effective in stimulating increased growth, whereas added sodium or potassium sulfates were not very stimulating. The results suggest that beneficial effect of added sodium chloride was due to the chloride rather than the sodium.

Wallace *et al.* (95), Van Itallie (90), and Lehr (40) have pointed out that the importance of sodium in plant nutrition may lie in its contribution of cationic balance within the plant.

Cationic balance.—Numerous recent contributions (6, 22, 52, 93, 95) have emphasized the tendency for the summation of the cation equivalencies per unit weight of dry matter to be constant

for a given species over a wide range of mineral nutrition. Interest in this concept was stimulated by the contribution of Van Itallie (89) showing the prevalence of the cation-equivalent constancy in Italian rye grass over extensive variations in the qualitative cation saturation of the soil. A later study with oats (90) provided substantiating data, and the weight of the evidence indicates that under a given environment at a given stage of ontogeny, the concept of cation-equivalent constancy is valid over a considerable latitude in mineral nutrition. Thus, variations in the content of one cation in the plant are accompanied by inverse variations in complementary cations. This has stimulated numerous investigators (6, 43, 71, 74, 79, 90) to ascertain the critical values of various cationic ratios which may have a bearing on plant response. Bear & Toth (6) report that when the calcium: potassium ratio in alfalfa tops exceeds four, or the potassium content drops below 1 per cent, alfalfa will respond to added potassium. Rogers (71) found that the yield of runner peanuts nearly quadrupled with an increase in the value of the calcium: potassium ratio from 1.7 to 3.0 in the vines. Lucas & Scarseth (43) and Stanford *et al.* (79) emphasize the importance of the calcium+magnesium: potassium ratio in corn stover as an index of yield potential. When the value of this ratio exceeded 6.0, poor yields of corn were invariably observed regardless of the actual levels of the cations.

There is no question but what "antagonism" or "counterbalancing effects" between various cations within the plant are highly important in conditioning metabolic activity. Goodall & Gregory (26) have made an exhaustive survey of the literature on the chemical composition of plants as related to their nutritional status and have presented a critical analysis of their findings. They state:

It is not to be denied that the ratios of nutrients within the plant may sometimes give useful indications as a supplement to those derived from the actual concentrations—though no information that could not be obtained from a curvilinear multiple regression—, but to use such ratios without consideration of the individual concentration data is, in most cases, unjustified.

They point out that the procedure involves certain hidden assumptions:

Let us suppose a three-dimensional figure in which the vertical axis represented whatever feature of development was being measured . . . while the two horizontal axes represented the content of the two nutrients in question in the plant material, all other factors being held constant; if development of the plant de-

depends on the ratio of the two nutrients within it, and not on their individual values, then any vertical plane through the origin must cut the figure in a horizontal line. This has never been demonstrated.

Cain (19) concurs with the conclusions of Goodall & Gregory (26) to the effect that no satisfactory mathematical expression of the interrelationships among the nutrient cations has been devised, even though a general interrelationship is apparent. Cain (19) points out on the basis of observations on apple trees that it is difficult to state whether magnesium deficiency symptoms are due to inadequate magnesium, or, in some instances at least, to an excess of potassium.

Progress in assessing the physiological role of the cations will probably continue to develop through cognizance of a concept of "balance" among the cations; not by calculating mathematical ratios of cations within the gross herbage of the plant, but by assaying the effect of relative cationic activities upon specific enzyme systems within the plant. For example, Braverman & Morgulis (13) have shown that there is a hyperbolic relationship between the hydrolytic action of actomyosin on adenosinetriphosphate and the magnesium: calcium ratio in the substrate. Over a wide range of magnesium: calcium ratios, actomyosin is almost completely inhibited, but at magnesium: calcium ratios characterized by low magnesium concentrations, activity of actomyosin increases rapidly with small decrease in magnesium:calcium. It was found that the action of magnesium ions on actomyosin also depends upon the concentration of potassium in the medium; magnesium enhanced enzymatic activity in the presence of 0.01 *M* potassium but is inhibitory at the 0.1 *M* level. Mommaerts & Seraidarian (55) have presented comparable data. Muntz (57) found that either potassium or ammonium is necessary in the substrate for the formation of hexosediphosphate from hexosemonophosphate as catalyzed by a dialyzed yeast maceration juice. Many other instances could be cited as to the specific effect on the nutrient cations in conditioning enzymatic process. The application of these studies to plant nutrition is manifest when one considers that plant growth is largely the resultant of enzymatically controlled energy transfers.

Nitrogen.—Nightingale (59) has recently reviewed the nitrogen nutrition of green plants, and no further elaboration is merited at this time.

Phosphorus.—Two factors are contributing to a rapid advancement in the understanding of phosphorus metabolism in plants: (a) the vast fund of information being accumulated with respect to phosphorylation in carbohydrate metabolism, and (b) the facility with which radioactive phosphorus may be used in tracer studies. Discussion of the phosphorylations involved in energy release through oxidation of glucose is beyond the scope of this review. Nevertheless, it should be mentioned that Aronoff & Calvin (3) used radiophosphate to test the theories to the effect that the reducing power of photosynthesis is utilized not only for the reduction of carbon dioxide but also, by means of coupled oxidations, for the generation of high energy phosphate bonds, or even high energy phosphate. They were not able to establish any direct connection between gross formation of organic phosphorus compounds and photosynthesis or photochemical reductions.

Movement of phosphate into and through the plant is readily studied by use of P^{32} . Ulrich *et al.* (88) found radiophosphorus in all aerial portions of grape vines within 40 hr. after it was applied to the soil, especially if it were applied in holes 15 inches deep near the trunk. The phosphorus tended to move most rapidly into the growing parts; the highest concentration appeared in the growing points and to a lesser degree in the bunches of young grapes. By use of this technic, the movement of considerable amounts of applied phosphorus into the grape vines was demonstrated, whereas macroanalysis of the vine parts before and after phosphate fertilization did not show a significant increase in phosphorus within the plant. This observation reflects on the validity of certain routine tissue tests.

Tuevaa & Samoilova (86) report that synthesis of nucleoproteins in squash is not inhibited by phosphorus deficiency but is actually enhanced. They noted that plants placed on adequate supply of phosphorus after having been subjected to phosphorus deficiency develop leaves and shoots of subnormal size suggesting symptoms of intoxication. They suggest that phosphorus deficiency induced a disturbance in metabolism which gave rise to toxic products within the plant. Laustalot & Winters (42) found that there was no consistent effect of phosphorus on the quinine content of *Cinchona ledgeriana*, but there was a tendency for total alkaloids to be higher in plants with a high phosphorus level. Mikhailov (53) reported that the rubber content of kok-saghyz was

50 per cent higher when the plants were grown with adequate as compared with inadequate levels of phosphorus. These reports on the relationship between phosphorus nutrition and the accumulations of certain end products in plants presumably take place through the effect of phosphorus on nitrogen and carbohydrate metabolism.

There have been numerous suggestions in the past that the phosphorus nutrition of plants is inhibited by the presence of soluble aluminum in the soil. Wallihan (97) has re-examined this concept and ascertained what effect the presence of aluminum in the nutrient medium might have in altering the metabolic activity of phosphorus in the tops of Ladino clover plants. He found no evidence that aluminum and phosphate are precipitated internally as aluminum phosphate and concluded that aluminum did not interfere to any measurable extent with the metabolic activity of phosphorus.

Boron.—Evidence is accumulating that the role of boron in plant metabolism is in some manner related to the activity of the oxidative enzymes. MacVicar & Burris (47) observed a marked increase in polyphenoloxidase activity in boron-deficient plant tissue. They suggested that the enhanced oxidation may be one of the causes of decreased carbohydrate content of apical tissues of boron-deficient plants. Reed (67) has also observed that the level of oxidase activity is higher in plant tissue afflicted with boron deficiency. By means of a cytochemical technic Reed (67) found that the cells of affected tissues of boron-deficient plants were invariably characterized by accumulations of phenolic precipitates in olive, and melanitic precipitates in celery. He deduced that the phenolic material that appeared in the afflicted olive cells indicated the irreversible oxidation of dihydroxyphenols as a result of inactivation of hydrogenases. That is, the normal equilibrium between the reduced and oxidized condition of the dihydroxyphenols (chiefly catechol) is destroyed. The catechol is oxidized to quinones which polymerize into the observed aggregates. He suggested that the hydrogenase system of the cell fails owing either to the blocking of the catalyst or to the lack of hydrogen donors or both. Reed has presented a fundamental approach to an understanding of the role of boron in plant metabolism, and this line of investigation should be amplified.

The earlier observation of Warington (99) to the effect that

the boron requirement of plants was lower under short days has been amplified by Struckmeyer & MacVicar (82). They found that the short-day plants, Biloxi soybean and cocklebur, fruited and showed virtually no symptoms of boron deficiency when exposed to short days on a boron-deficient nutrient, whereas they developed marked symptoms on the same nutrient under long days. Day neutral plants, tomato and sunflower, showed striking symptoms of boron deficiency regardless of day length. Struckmeyer & MacVicar suggest that the decrease in cambial activity associated with early floral induction or photoperiodic induction was related to the decreased severity of symptoms of boron deficiency on short-day plants under short days.

MacVicar & Tottingham (46) were unable to confirm a previous observation by Eaton (25) that an application of indoleacetic acid will partially replace boron as an essential element.

Indications of relationships between level of boron nutrition and that of other elements continue to appear. Brennan & Shive (15) have presented further evidence of the reciprocal effects of calcium and boron. Boron deficiency symptoms on tomato were most pronounced at a high level of calcium supply; whereas boron toxicity symptoms were most pronounced at a relatively low level of calcium supply. Shear *et al.* (75) observed that the severity of boron toxicity symptoms in tung trees may be related to the magnesium:boron ratio. It is doubtful that the various ratios between boron and nutrient cations are effective per se in plant metabolism, and they become apparent owing to the differential effect of boron and other nutrients upon specific enzyme systems.

Iron, copper, and manganese.—Hewitt (31) studied the iron status in sugar beets in relation to manganese and other metals towards evaluation of the hypothesis of Somers & Shive (78) to the effect that basis for the observed interrelationship between iron and manganese in plants rested with the respective redox potentials of these two metals. Hewitt (31) reported that chlorosis typical of iron deficiency developed on supplying a given level of various ions in the following decreasing order of severity: cobalt, copper, zinc and chromate, nickel, chromic, manganous, lead. The results showed no relation to redox potential. Hewitt concluded that, (a) manganese is not unique in its ability to induce iron-deficiency symptoms; (b) simultaneous deficiencies of iron and man-

ganese may be observed in the same plant thus showing some independent functions; (c) toxic effects of excess manganese can be readily distinguished from iron deficiency, again suggesting independent effects; and (d) the hypothesis based on oxidation-reduction potentials of simple ions appears inadequate to explain the effects of the above-mentioned metals in inducing iron-deficiency symptoms.

The chlorosis of leaves arising from a disturbance in iron metabolism may be associated with a wide diversity of conditions in the substrate. The prevalence of iron chlorosis of plants on calcareous soil is frequently observed, but it is indeed unusual to find iron chlorosis developing on plants growing on an acid peat soil. Walsh & Clarke (98) have observed such a condition in tomato plants. The chlorosis was associated with a low content of iron in the leaves and was corrected by spraying with ferrous sulfate. These investigators found no evidence of immobilization of iron in the plants. They found that the peat medium contained adequate amounts of iron and considerable amounts of soluble zinc, especially where chlorosis was severe. It was suggested that the relatively high level of zinc probably impaired root activity and interfered with transference of iron from roots to leaves. Jones & Tio (36) have reported frenching of tobacco associated with low iron content of plants growing on a slightly acid greenhouse compost. Frenching developed on plants grown at a soil temperature of 35°C. It failed to develop when the soil temperature was maintained at 21°C. and was prevented by autoclaving the soil prior to growing the plants at a root temperature of 35°C. Addition of ferrous sulfate and ferric phosphate to a frenching soil also prevented development of the symptoms. The results indicate that the high soil temperature encouraged certain microbiological activity which inhibited the normal availability of iron from the soil to the plant. As Burström (18) points out, the relation of bacteria to iron in the soil is very imperfectly known.

Zinc.—The relationship between zinc and auxin in plant growth first set forth by Skoog (76), has been further amplified by Tsui (84, 85). He found (84) that the auxin decrease in zinc-deficient tomato plants occurred before there was any decrease in growth, or other noticeable symptoms. When zinc was added to the nutrient solution auxin in the plant increased within a few days. Synthetic L-tryptophane was converted to active growth substance by leaf

discs with equal facility from both control and zinc-deficient plants. A decreased content of tryptophane was noted in zinc-deficient plants even before visible symptoms were evident; and tryptophane increased on the addition of zinc. In view of the observations of Wildman *et al.* (101) that tryptophane is a precursor of indoleacetic acid, Tsui concluded that zinc is required directly for the synthesis of tryptophane and indirectly for the synthesis of auxins. This work represents commendable progress in ascertaining the metabolic role of a nutrient element. Tsui (85) also noted that there was a significant decrease in water content and an increase in the osmotic pressure of the tissue fluids of the tops of zinc-deficient plants before there were any visible symptoms of zinc deficiency.

Bergh (8) found a much wider variation in the zinc content of the dry matter of the straw than of the grain of cereals grown under varying levels of zinc supply. For example, the zinc in wheat straw was found to vary from 15 to 416 p.p.m. of the dry matter, whereas zinc content of the grain from the same group of treatments varied from 57 to 157 p.p.m. There appeared to be marked differences among the cereals as to the zinc requirement for maximum yield. The best yield of oats was on the treatment giving lowest zinc content of the grain, 40 p.p.m. The highest yield of barley was associated with a relatively high zinc content of the grain, 132 p.p.m.

Molybdenum.—Hewitt & Jones (30), Mulder (56), and Stout & Meagher (81) have presented evidence that molybdenum is in some manner related to nitrate reduction in plants, since nitrate is not efficiently reduced in molybdenum-deficient plants. The high accumulation of nitrate in plants afflicted with molybdenum deficiency may be rapidly dissipated by an injection or addition of molybdenum. Mulder (56) noted the symptoms of molybdenum deficiency were much less severe when the plants were supplied with ammonium nitrogen as compared with nitrate nitrogen.

Using radioactive Mo^{98} and Mo^{99} in short-time absorption experiments with tomato plants, Stout & Meagher (81) were able to follow the rate of entry of molybdenum into the plant and its distribution therein. Roots accumulated molybdenum rapidly from the culture solution even though the initial concentration of this element was one part per billion. Radioautographs showed that the distribution of molybdenum in the leaves follows a dif-

ferent type of pattern from that of other mineral nutrients. This element does not accumulate rapidly in actively metabolizing plant cells adjacent to the vascular tissue; but it does accumulate in areas of the leaf having the greatest number of stomatal openings. The amount of molybdenum taken in by the plant is markedly influenced by the concentration of the phosphate ion in the nutrient medium. Walker (92) has demonstrated the presence of molybdenum deficiency on barren serpentine soil.

MINERAL CONTENT OF PLANTS IN RELATION TO GROWTH RESPONSES

Investigators using chemical composition of plants as a method of nutritional diagnosis have benefited greatly by the extensive compilation and review by Goodall & Gregory (26). They summarize the value of plant analysis as a diagnostic method as follows: (a) plant analyses are much more useful than soil analyses because of the difficulty of obtaining reliable soil samples; (b) chemical analyses are preferable to deficiency symptoms because the latter are not always specific and crop growth is usually impaired before symptoms develop, but the incipency of nutrient inadequacy could early be detected by chemical means; and (c) plant analysis is much more economical than field trials, but its validity must be based on capacity to indicate field response. The latter contingency is not all that may be desired. Although chemical analysis can provide a sensitive indication of the need for elements in relatively poor supply, it is not sensitive to changes in level of nutrition of elements adequate in relation to other "limiting factors." In general, analysis often shows only which element is "limiting," and not those to which a response would be obtained if the supply of the "limiting" element were itself increased.

There have been several recent reports which are rather discouraging in their evaluation of plant tissue testing as a diagnostic method. Chubb & Atkinson (21) and Atkinson *et al.* (4) were unable to find any satisfactory relationship between tissue tests and crop yields. Where crops responded to nitrate, tissue testing was of some value. Wolf & Ichisaka (102) also concluded that the usefulness of plant analysis as a guide to the fertilizer needs of spinach was rather limited. The test for nitrogen was found to be the one of most value. Reuther (68) found no significant relationship between the mineral composition of date palm pinnae and the vegetative vigor of the trees.

One could reasonably postulate that for any given combination of environmental factors, there is within a given plant tissue an optimum content and relationship of the mineral nutrients for maximum plant growth; and that deviations from this optimum nutritional status would be accompanied by decreases from maximum growth. This idealized thesis sets forth the theoretical basis of plant analysis as a diagnostic method, but it is probably not experimentally verifiable because of the extreme complexity of pertaining factors, and because of the evidence that growth response is invariant over a considerable range in content of many of the nutrient elements. Irrespective of negative observations, it is evident that chemical analysis will continue to be an important diagnostic aid under many conditions. As Goodall & Gregory (26) point out, if the investigator works out a satisfactory technic that takes into account the selection of the samples and the physiological stage of development of the given variety of a crop under the prevailing weather conditions, plant analysis can be of great value in predicting the fertilizer needs of plants, particularly for perennial crops. Tyner (87), Hill & Cannon (32), and Kenworthy & Gilligan (38) have recently shown significant relationships between tissue analyses and growth response of crops. They show that the relations are by no means simple, however. For example, growth response was found to be positive over the lower range of content of tissue phosphorus, but negative over the higher range; and this effect was conditioned by the level of nitrogen and potassium in the tissue. Laustalot & Winters (42) have presented supporting evidence, especially with respect to the nitrogen-phosphorus interrelationship.

The value of plant tissue tests will be greatly enhanced as information accrues on the normal trends in mineral content of various plant tissues with the ontogeny of the plant. Sayre (73) has presented such information on the corn plant as a result of very extensive experiments. He found that nitrogen, phosphorus, and potassium entered the corn plant and moved from tissue to tissue independently. Nitrogen accumulation reached a maximum at silking time and ceased about four weeks later in the season studied. Nitrogen continued to move into the grain from other tissues until maturity. Phosphorus accumulation did not cease until about maturity, and this element also moved into the grain from other tissues. Potassium accumulation reached a maximum about three weeks after silking, and there was an actual loss of potassium

after that time largely from the leaves and stems. There was no accumulation of potassium in the grain.

Cain & Boynton (20) determined the mineral content of mid-shoot apple leaves over the growing season. Nitrogen decreased continuously from the maximum observed in May to October. Phosphorus showed little variation except for a slight negative trend with time. Potassium reached a maximum in the leaves by June 1 and then showed a gradual decrease until leaf-fall in October. Calcium increased markedly in the leaves as the season progressed. Olsen (60) presented very similar observations on the leaves of beech trees. Cain & Boynton (20) found also that the curves depicting the seasonal trend of a given element in apple leaves may be displaced depending on the load of fruit carried by the tree and upon the level of nitrogen fertilization. It is obvious that valid use of tissue testing as a diagnostic procedure depends on a consideration of the developmental stage of the plant and prevailing environmental conditions.

EXPERIMENTAL DESIGN

There is a manifest need for agreement among investigators dealing with the mineral nutrition of plants as to the advantages and limitations of the various methods of inducing variation in artificial substrates. There is no way of varying the concentration of an ion in a nutrient solution without concomitant variation in the concentration of the "balancing ion." This "balancing ion," frequently sodium or chloride, is assumed to be without effect in the response to the primary variant. The plant may not be fully conversant with this assumption on the part of the investigator. Leonard *et al.* (41) grew sweet potatoes at various nutrient levels. The response to nitrogen was studied over a range in supply from 10 to 800 p.p.m. and this variation in nitrogen was accompanied by a range in sodium concentration from 96 to 1,380 p.p.m. Calculations indicated that the osmotic pressure of these solutions in the nitrogen series varied from about 0.7 atm. to about 3.0 atm. There was no certain way of segregating the effect of nitrogen from that of sodium or osmotic pressure. The calcium supply was varied from 10 to 1,000 p.p.m. and the chloride ion was concurrently varied from 110 to 1,880 p.p.m. It may be shown that the osmotic pressure also varied from about 0.8 to about 3.0 atm. Thus, the observed response cannot be attributed merely to level of calcium

supply. The wide range in the levels of the "balancing ions" undoubtedly had a marked effect in this experiment, but it was not possible to evaluate the effect in relation to that of the nutrient ion primarily varied. Leonard *et al.* (41) recognized this difficulty in interpreting their results.

Weidemann & Cook (100) present a system of "balanced nutrient solutions" in which it is supposedly possible to omit one of the nutrient ions without disturbing the "balance" of the other ions. This is accomplished by increasing the level of the sulfate ion in the nitrogen deficient solutions and by varying the proportion of ammonium to nitrate in the nitrogen supply when other elements are omitted. Cain (19) also varied the proportion of ammonium to nitrate in his nitrogen supply to effect ionic "balance" in studying the influence of varying proportions of the mineral cations on the nutrition of young apple trees. There is no escaping the fact that the ammonium is very much a nutrient cation and its presence cannot be ignored in assaying the effect of cationic balance on plant response. Furthermore, it is indeed dubious that wide variations in ammonium:nitrate ratio may be regarded as equivalence in nitrogen nutrition. If it is necessary to incorporate this condition into the experimental design, some indication should be given as to the effect of varying ammonium:nitrate ratio per se.

No assumptions need be made concerning balancing ions in triangular designs permitting independent variation of anions and cations as first described by Beckenbach *et al.* (7); all ions are an integral part of the set-up. As Richards (69) points out, however, this design does not submit to factorial analysis and effects which may be correlated with variations in one cation are also inversely correlative with the sum of the other two cations. The latter objection is not too serious unless one permits statistics to take the place of logic. A tetrahedral design which would permit inclusion of sodium and chloride as supposedly inert ions would seem to have many advantages. Total concentration would be maintained relatively constant, an almost endless variety of ionic proportions could be selected, and treatments could be chosen which would permit factorial analysis.

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